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Bacteria and Extracellular Polymeric Substances in Activated Sludge Scum Formation

Elisabeth Müller

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1. Univ.-Prof. Dr.-Ing., Dr. h. c. Peter A. Wilderer, i. R.
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3. Univ.-Prof. Dr. rer. nat. Harald Horn

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Schön ist alles, was Himmel und Erde verbindet:

der Regenbogen,

die Sternschnuppe,

der Tau,

die Schneeflocke,

doch am schönsten ist das Lächeln eines Kindes.

Für meine Tochter

Mira Madgalena

For my daughter

Mira Madgalena

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ABSTRACT

The formation of thick viscous scum layers is a persistent problem in wastewater treatment plants (WWTPs) observed all over the world. Three factors are necessary for scum formation: (i) dispersed gas bubbles, (ii) surface-active material and (iii) hydrophobic compounds. For instance, air bubbles in the aeration tank initialize scum development. The gas-water interface is stabilized by the adsorption of surface-active molecules. The presence of hydrophobic material provides stable adhesion to solid particles promoting persistent scum layers. Surface-active substances may enter the WWTP via primary effluent or be produced by various bacteria in the presence of hydrophobic carbon sources and under nutrient deficiency. Hydrophobic material might originate from the primary effluent or be available by an abundant growth of organisms with hydrophobic cell surfaces.

One objective of this study was to characterize the relevant bacteria that are involved in scum formation. The identification of these bacteria was performed by classical microscopic sludge analysis and molecular biological methods to obtain information about their taxonomic affiliation. The knowledge about the phylogeny of scum bacteria might reveal physiological properties of these bacteria, which is helpful for the understanding of the scum formation process and the application of specific control measures. A further topic of this study was to investigate whether scum formation is a passive floating mechanism of activated sludge to the water surface or an active growth process of a specific scum biocenosis close to the water surface. The elucidation of the role of these scum bacteria and extracellular polymeric substances (EPS) in the formation of stable scum layers was the third objective of this work. EPS might perform two different functions in the scum process: (i) they serve as nutrient source and promote the growth of a specific bacterial biocenosis and (ii) they directly stabilize scum layers due to their hydrophobic sites. For this purpose, scum bacteria numbers quantified by fluorescence in situ hybridization (FISH) were correlated to sludge hydrophobicity and specific EPS components extracted from activated sludge and scum.

A screening of various WWTPs with scum formation revealed that in most plants examined *Microthrix parvicella* dominated the activated sludge and scum biocenoses. Furthermore nocardioform actinomycetes could be characterized as important scum bacteria. These microorganisms were found at very low numbers in activated sludge whereas they were clearly enriched in scum layers. The filamentous type 1863 found in several WWTPs was clearly enriched in the scum fraction but never detected as the dominant microorganism within the biocenosis. The filamentous bacterial morphotypes 1851, 0041/0675, 0092, and *Nostocoida limicola* were identified only in some WWTPs as the dominant organisms. However, they were often found concomitantly in *M. parvicella*- and nocardioform actinomycetes-dominated biocenoses. The identification of filamentous bacterial morphotypes by classical microscopy often failed because of morphological and taxonomic variations. Therefore the FISH technique using specific rRNA-targeted oligonucleotide probes was applied to reveal reliable phylogenetic information on these described morphotypes. The effectiveness of the two methods, classical sludge analysis and FISH, for detection, identification, and quantification of filamentous organisms in activated sludge and scum was compared. Classical sludge analysis and FISH corresponded well in the case of filamentous *M. parvicella*, which was identified as "*Candidatus* Microthrix parvicella", an unclassified member of the *Actinobacteria*. Type 1863 was characterized as *Acinetobacter* spp. and type 1851 was identified as a member of the *Chloroflexi* subdivision 3, closely related to

Roseiflexus castenholzii. Classical microscopy overlooked nocardioform actinomycetes and type 1863 single cells, which FISH detected only. Furthermore the non-branched filamentous nocardioform actinomycetes growing as short filaments or in cell clusters were clearly identified by FISH only. FISH underestimated branched filamentous nocardioform actinomycetes and morphotypes 0041/0675 or 0092 because of insufficient cell wall permeability for fluorescently labeled rRNA-targeted oligonucleotide probes, and also because of their taxonomic variability and consequently the availability of adequate probes. FISH results for *N. limicola* morphotypes I and III are still insufficient because of both their low in situ numbers and due to their high taxonomic variability. However, morphotype *N. limicola* II was frequently identified as "*Candidatus* Nostocoida limicola" belonging to the *Actinobacteria* phylum.

The identification of the nocardioform actinomycetes at genus and species level was limited by classical microscopy because the different species exhibit mostly the same morphotype. The use of FISH for this purpose was restricted due to the deficit of available genera- and species-specific probes. The application of the full-cycle rRNA approach, combined with nucleic acid fingerprinting methods, restriction fragment length polymorphism (RFLP) and denaturing gradient gel electrophoresis (DGGE), proved to be a successful approach to identify unknown nocardioform actinomycete species in different scum samples. A screening of the clone libraries with *Actinobacteria*-specific primers followed by RFLP profiling allowed to organize clone libraries into taxonomic groups and the identification of the dominant clones for each library. The analysis of environmental DNA by DGGE and the comparison with DGGE patterns of the dominant clones indicate that the dominant clone insert represented the dominant species within the *Actinobacteria* phylum of the microbial scum communities analyzed. However, only the sequence analysis of clone inserts followed by probe design and the application of FISH using specific probes for *Dietzia* spp. and various *Rhodococcus* spp. (*R. erythropolis* and probably two new species within the *Rhodococcus* radiation) verified and quantified the presence of these organisms. FISH quantification revealed that these different *Rhodococcus* species, characterized by a typical branched filamentous morphotype (GALO), were found in high numbers in the scum fraction originating from German WWTPs. They were often accompanied by *Dietzia* spp. growing as non-branched short filaments or in cell clusters generally found in lower numbers. In contrast, scum samples in Australian WWTPs were dominated by *Gordonia* spp. and *Skermania piniformis*. The occurrence of the described nocardioform actinomycete species could be correlated with different growth conditions in the WWTPs concerning temperature and the availability of specific carbon sources.

Physicochemical analyses such as hydrophobicity measurements and EPS characterization combined with FISH quantification of the dominant scum bacteria were performed on sludge samples originating from *M. parvicella*- and nocardioform actinomycetes-dominated plants with scum events. In addition, respiratory activity was monitored in activated sludge and scum to define growth strategy and ecological niche of *M. parvicella* and nocardioform actinomycetes. Lipophilic substances originating from the primary effluent and being adsorbed to the activated sludge EPS promoted the growth rate of *M. parvicella*, which was shown to be more active in activated sludge as compared to scum. The long hydrophobic *M. parvicella* filaments enlarge and hydrophobize the surface structure of activated sludge flocs causing stable adhesion to gas bubbles. Thus these flocs are assumed to just passively float to

the water surface producing persistent scum layers. In contrast, a higher respiratory activity in the scum biocenosis as compared to the activated sludge biocenosis was determined in nocardioform actinomycetes-dominated WWTPs. Therefore nocardioform actinomycetes as was shown for several *Rhodococcus* species and *Dietzia* spp. might enhance their population density by actively growing in the floated sludge fraction. This is supported by the low numbers of nocardioform actinomycetes detected in activated sludge and the clear enrichment of these organisms in the scum fraction. The active growth process in the scum layer is genera- and species-dependent, promoted by different scum EPS compounds such as lipophilic substances or other not yet characterized substances in addition to proteins and carbohydrates. Both high numbers of *Rhodococcus* spp. and lipophilic substances on the water surface caused hydrophobicity and thus scum formation. The described different scum formation strategies lead to various control measures with *M. parvicella* to be controlled in the activated sludge fraction and nocardioform actinomycetes to be fought in the scum fraction. This study revealed that different nocardioform actinomycete species found in the scum layer require specific growth conditions. Therefore the troubleshooting measures for nocardioform actinomycetes-dominated scum should be based upon a reliable taxonomic identification at genus and species level.

ZUSAMMENFASSUNG

Die Bildung von dickem viskosem Schaum stellt ein häufiges Problem in Abwasserreinigungsanlagen (ARA) dar. Drei Faktoren spielen bei der Entwicklung von stabilen Schäumen eine wichtige Rolle: (i) fein verteilte Gasblasen, (ii) oberflächenaktive Substanzen und (iii) hydrophobe Stoffe. In der belüfteten Phase entstehen die Gasblasen z.B. durch Sauerstoffeintrag und initiieren die Schaumbildung. Die Grenzflächen zwischen der Gas- und Wasserphase werden durch Adsorption von oberflächenaktiven Substanzen stabilisiert. Das Vorkommen von hydrophoben Stoffen unterstützt die Adhäsion dieser Gasblasen an Feststoffpartikel, wodurch die Bildung von stabilen Schaumschichten gefördert wird. Oberflächenaktive Stoffe können über den Zulauf in die Anlage gelangen oder von verschiedenen Bakterien in der Anwesenheit von hydrophoben Kohlenstoffquellen bzw. unter Nährstoffmangel produziert werden. Hydrophobe Substanzen können aus dem Abwasser stammen oder werden durch das starke Wachstum von Organismen mit einer stark hydrophoben Zelloberfläche zur Verfügung gestellt.

Eine Zielsetzung dieser Studie war es, die relevanten Schaum-Bakterien, welche an der Schaumbildung beteiligt sind, zu charakterisieren. Die Identifizierung dieser Bakterien wurde anhand der klassischen mikroskopischen Schlammanalyse und molekularbiologischen Methoden, welche Information über die taxonomische Zugehörigkeit liefern, durchgeführt. Das Wissen über die Phylogenie der Schaum-Bakterien gibt Hinweise zu den physiologischen Eigenschaften dieser Bakterien, welche hilfreich sind für das Verstehen des Schaumbildungsprozesses und die Anwendung spezifischer Kontrollmaßnahmen. Eine weitere Frage dieser Arbeit war es zu untersuchen, inwieweit die Schaumbildung einen passiven Flotationsprozess des Belebtschlammes an die Wasseroberfläche oder einen aktiven Wachstumsprozess von spezifischen Bakterien an der Wasseroberfläche darstellt. Die Aufklärung der Rolle dieser Schaum-Bakterien und der extrazellulären polymeren Substanzen (EPS) bei der Bildung von stabilen Schäumen stellte den dritten Arbeitschwerpunkt dar. EPS erfüllen möglicherweise zwei verschiedene Funktionen im Schaumbildungsprozess: (i) sie dienen als Nährstoffquelle und fördern das Wachstum einer speziellen bakteriellen Biozönose und (ii) sie können aufgrund ihrer hydrophoben Oberflächen die Schaumschicht direkt stabilisieren. Zur Beantwortung dieser Fragen wurde die Anzahl der Schaum-Bakterien, die mit Hilfe der Fluoreszenz in situ Hybridisierung (FISH) quantifiziert wurde, mit der Schlammhydrophobizität und spezifischen EPS-Komponenten des Belebtschlammes und der Schaummatrix korreliert.

Die Screening-Untersuchungen an verschiedenen ARA mit Schaumereignissen zeigten, dass in den meisten untersuchten Anlagen *Microthrix parvicella* sowohl die Schlamm- als auch die Schaumbiozönosen dominierte. Weiter konnten nocardioforme Actinomyceten als wichtige Schaumbakterien charakterisiert werden. Diese Organismen wurden in geringer Anzahl im Belebtschlamm gefunden und waren in der Schaumschicht deutlich angereichert. Das fadenförmige Bakterium Typ 1863 wurde in mehreren ARA deutlich angereichert im Schaum gefunden, wurde aber niemals als dominanter Organismus innerhalb der Biozönose nachgewiesen. Die fadenförmigen Morphotypen 1851, 0041/0675, 0092 und *Nostocoida limicola* wurden nur in einigen ARA als die dominanten Organismen im Belebtschlamm identifiziert. Jedoch wurden sie häufig zusammen mit *M. parvicella*- und nocardioforme Actinomyceten-dominierten Biozönosen gefunden. Die Identifizierung von fadenförmigen

Bakterienmorphotypen ist häufig aufgrund von morphologischen Abweichungen und unvollständigen taxonomischen Informationen fehlerhaft. Deshalb wurde die FISH-Technik unter Verwendung von spezifischen rRNA-gerichteten Oligonukleotidsonden eingesetzt, um verlässliche phylogenetische Informationen über die beschriebenen Morphotypen zu erhalten. Die beiden Methoden, die klassische mikroskopische Schlammanalyse und FISH, wurden in Bezug auf ihre Effektivität zum Nachweis, zur Identifizierung und zur Quantifizierung fadenförmiger Organismen im Belebtschlamm und Schaum verglichen. Für den Morphotyp *M. parvicella*, welcher als „*Candidatus* Microthrix parvicella“ (ein nicht-klassifiziertes Bakterium der *Actinobacteria*) identifiziert wurde, stimmten die Ergebnisse der klassischen Schlammanalyse und FISH gut überein. Typ 1863 wurde als *Acinetobacter* spp. charakterisiert und Typ 1851 wurde als Bakterium der *Chloroflexi* Gruppe 3, nah verwandt mit *Roseiflexus castenholzii*, identifiziert. Die klassische Mikroskopie übersieht Einzelzellen der nocardioformen Actinomyceten und des Typs 1863, welche nur mit FISH nachgewiesen werden konnten. Weiterhin wurden unverzweigte nocardioforme Actinomyceten, die als kurze Fäden oder in Zellcluster wuchsen, nur mit FISH eindeutig identifiziert. Die FISH-Methode unterschätzt die Anzahl der verzweigten fadenförmigen nocardioformen Actinomyceten und der Morphotypen 0041/0675 oder 0092. Die Ursachen hierfür sind erstens die limitierte Zellwandpermeabilität für fluoreszenzmarkierte rRNA-gerichtete Oligonukleotidsonden und zweitens die Verfügbarkeit von geeigneten Sonden. Die FISH-Ergebnisse für den Morphotyp *N. limicola* I und III sind sowohl wegen ihrer geringen Anzahl als auch wegen ihrer taxonomischen Vielfalt wenig aussagekräftig. Der Morphotyp *N. limicola* II wurde jedoch häufig als „*Candidatus* Nostocoida limicola“ aus der Gruppe der *Actinobacteria* identifiziert.

Die Identifizierung der nocardioformen Actinomyceten auf Gattungs- und Artebene mit Hilfe der klassischen Mikroskopie war nicht möglich, da die verschiedenen Arten zumeist den gleichen Morphotyp aufweisen. Der Einsatz von FISH für diesen Zweck war aufgrund des Mangels an verfügbaren gattungs- und artspezifischen Sonden ebenso begrenzt. Die Anwendung des „full-cycle“ rRNA-Ansatzes in Kombination mit verschiedenen DNA-Fingerprint-Methoden, dem Restriktionsfragmentlängen-Polymorphismus (RFLP) und der denaturierenden Gradienten-Gelelektrophorese (DGGE) erwies sich als eine erfolgreiche Methode zur Identifizierung der unbekannten nocardioformen Actinomyceten-Arten in den verschiedenen Schaumproben. Das Screening der Klonbibliotheken mit *Actinobacteria*-spezifischen Primern gefolgt von der Erstellung von RFLP-Profilen machte es möglich, die Klone in taxonomische Gruppen zu ordnen, und erlaubte die Identifizierung der dominanten Klone der einzelnen Klonbibliotheken. Die Analysen der Umwelt-DNA mit Hilfe der DGGE und der Vergleich zu den DGGE-Mustern der dominanten Klone wiesen darauf hin, dass die dominanten Klon-Inserte die dominanten Bakterien innerhalb der *Actinobacteria* der untersuchten mikrobiellen Schaumbiozönosen repräsentierten. Jedoch nur die Sequenzanalyse von Klon-Inserten gefolgt von Sondendesign und die Anwendung von FISH mit spezifischen Sonden für *Dietzia* spp. und verschiedenen *Rhodococcus* spp. (*Rhodococcus erythropolis* und wahrscheinlich zwei neue Arten der Gattung *Rhodococcus*) bestätigten und quantifizierten das Vorkommen dieser Organismen. Die FISH-Quantifizierung zeigte, dass die verschiedenen *Rhodococcus*-Arten, welche durch den typisch verzweigten fadenförmigen Morphotyp (GALO) charakterisiert waren, in hoher Anzahl in der Schaumfraktion der deutschen ARA vorkamen. Sie traten häufig zusammen mit *Dietzia* spp. auf, welche als kurze unverzweigte

Fäden bzw. in Zellcluster wuchsen und im Allgemeinen in geringerer Anzahl gefunden wurden. Im Gegensatz dazu dominierten in den Schaumproben der australischen ARA *Gordonia* spp. und *Skermania piniformis*. Das Vorkommen der beschriebenen nocardioformen Actinomyceten-Arten konnte mit den verschiedenen Wachstumsbedingungen der ARA, welche Temperatur und die Verfügbarkeit von spezifischen Kohlenstoffquellen betreffen, korreliert werden.

Die physikochemischen Analysen, welche die Messung der Hydrophobizität und die Charakterisierung der EPS umfassen, und die FISH-Quantifizierung der dominanten Schaum-Bakterien wurde an verschiedenen Schlammproben durchgeführt. Diese Proben stammten aus *M. parvicella*- und nocardioforme Actinomyceten-dominierten Anlagen mit Schaum-ereignissen. Zusätzlich wurde die Stoffwechselaktivität im Belebtschlamm und Schaum erfasst, um die Wachstumsstrategien und die ökologischen Nischen von *M. parvicella* und nocardioformen Actinomyceten zu bestimmen. Die höhere Stoffwechselaktivität der *M. parvicella*-dominierten Belebtschlammbiozönose im Vergleich zur Schaumbiozönose weist darauf hin, dass das Wachstum von *M. parvicella* im Belebtschlamm stattfindet. Die Wachstumsraten von *M. parvicella* werden durch die lipophilen Stoffe, welche aus dem Abwasser stammen und an die Belebtschlamm-EPS adsorbieren, gefördert. Die langen hydrophoben *M. parvicella*-Fäden vergrößern und hydrophobieren die Oberflächenstruktur der Belebtschlammflocke, wodurch eine stabile Adhäsion an Gasblasen stattfindet. Dadurch besitzen diese Flocken die Fähigkeit passiv an die Wasseroberfläche zu flotieren und stabile Schaumschichten zu entwickeln. Im Gegensatz dazu wurde eine höhere Stoffwechselaktivität in der Schaumbiozönose im Vergleich zur Belebtschlammbiozönose in nocardioformen Actinomyceten-dominierten ARA bestimmt. Nocardioforme Actinomyceten vergrößern ihre Populationsdichte durch aktives Wachstum in der flotierenden Schlammschicht, was für verschiedene *Rhodococcus*-Arten und *Dietzia* spp. gezeigt wurde. Diese Beobachtung wurde durch die geringe Anzahl der nocardioformen Actinomyceten im Belebtschlamm und die deutliche Anreicherung dieser Organismus in der Schaumschicht bestätigt. Der aktive Wachstumsprozess in der Schaumschicht wird von verschiedenen EPS-Komponenten wie z.B. den lipophilen Stoffe und anderen noch nicht charakterisierten Substanzen gefördert. Sowohl die hohe Anzahl von *Rhodococcus* spp. als auch lipophile Stoffe an der Wasseroberfläche bewirken eine Zunahme der Hydrophobizität und folglich die Entwicklung von stabilen Schäumen. Die beschriebenen Strategien zur Schaumbildung benötigen verschiedene Kontrollmaßnahmen, wobei *M. parvicella* direkt im Belebtschlamm bekämpft werden muss und die nocardioformen Actinomyceten in der Schaumschicht kontrolliert werden müssen. Diese Studie zeigt, dass verschiedene nocardioforme Actinomyceten-Arten, welche in der Schaumschicht nachgewiesen wurden, spezifische Wachstumsbedingungen benötigen. Deshalb sollten Bekämpfungsmaßnahmen für nocardioforme Actinomyceten-dominierte Schäume auf einer zuverlässigen taxonomischen Identifizierung auf Gattungs- und Artebene basieren.

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1 INTRODUCTION

Wastewater is used water, which is characterized by a high variation of pollutants depending on origin. The major contaminants found in wastewater are biodegradable organics, volatile organic compounds, toxic metals, suspended solids, nutrients (nitrogen and phosphorus) and microbial pathogens. In order to protect the environment these contaminants are to be removed before wastewater is discharged into any surface waters. Wastewater purification includes different treatments based on mechanical, biological and chemical processes. Mechanical treatment removes large objects. Microorganisms degrade or store dissolved and particulate contaminants during biological treatment, and chemical treatment converts dissolved substances such as phosphorus into a solid, settleable form by precipitation with iron or aluminum compounds. The mostly used technology for the biological treatment of municipal wastewater is the activated sludge system characterized by a suspended growth of microorganisms, forming activated sludge flocs. The separation of the purified water from activated sludge flocs by sedimentation represents the last and crucial step of the activated sludge process before the treated effluent is discharged into the environment. There are several types of problems regarding solid separation in activated sludge systems leading to deterioration of the effluent quality. The main known operating problems are (i) bulking due to the overgrowth of filamentous bacteria leading to voluminous activated sludge and (ii) scum formation known as well as foaming, which is characterized by the development of a stable viscous biomass layer floating on the water surface of the aeration tank and/or clarifier as well as digester.

Scum formation in the activated sludge process is a common problem encountered in many wastewater treatment plants (WWTPs) around the world. A survey study performed 1995/1996 on German municipal WWTPs (PE > 10,000) indicated that 75% of the 83 WWTPs examined suffered from scum at least once per year (Lind and Lemmer 1998). The phenomenon of biological scum causing operational troubles is not new and was described for the first time in 1969 as the “Milwaukee mystery” observed at the Jones Island East WWTP in Milwaukee, USA (Anonymous 1969). Since that time many research groups dealt with the problem of scum formation and tried to understand the processes causing the development of a stable scum layer. The first report about scum biocenoses showed that Gram-positive branching actinomycetes identified as *Nocardia amarae* were present in high numbers (Lechevalier and Lechevalier 1974). Since then the problem has often been described as *Nocardia* foam, although this term is not correct as later observations demonstrated that many other filamentous organisms are involved in scum formation. Furthermore *Nocardia amarae* was meanwhile reclassified as *Gordonia amarae* and according to the new taxonomic classification no *Nocardia* spp. were ever detected in WWTPs with scum events.

Most of the current reports on scum research bring to focus the characterization of the bacterial populations involved. It was shown that the dominant scum bacteria comprise the filamentous organisms *Microthrix parvicella*, nocardioform actinomycetes, *Nostocoida limicola*, and Eikelboom types 0041/0675, 1851, and 0092 (Lind and Lemmer 1998). Moreover physicochemical analyses such as hydrophobicity measurements and surface charge analyses were correlated with the presence of defined scum bacteria (Stratton et al. 1998, Hladikova et al. 2002). However, still little is known about the exact role of these bacteria in

the scum process. Furthermore in most survey studies about the dominant scum bacteria the identification of these bacteria is based on their morphology and different staining behavior, which often gives not enough information about their taxonomic affiliation. The phylogeny of scum bacteria might give an indication about their physiological activities in the scum layer, which is very important for effective control strategies.

This study focused on the identification of dominant scum bacteria by their morphology and compared these data with the phylogenetic characterization using different molecular biological methods. The application of fluorescence in situ hybridization (FISH) using specific probes for filamentous bacteria, DGGE technique, and the full-cycle rRNA approach revealed new phylogenetic knowledge about scum bacteria. The last topic was to correlate scum bacteria numbers analyzed by quantitative FISH with parameters such as sludge hydrophobicity and the chemistry of extracellular polymeric substances (EPS) in both activated sludge and scum. The aim was to obtain information about (i) the nutrient conditions in the sludge and scum matrix causing selective growth conditions for scum bacteria and (ii) the impact of scum bacteria and/or EPS on the hydrophobic properties of sludge and scum, and consequently on scum development.

2 STATE OF KNOWLEDGE

2.1 Activated Sludge Wastewater Treatment

The activated sludge wastewater process allows very effective treatment of municipal wastewater and is hence the most widespread technology for wastewater purification. This technology was invented and first reported by English chemists at the beginning of the 20th century (Ardern and Lockett 1914a, b). The conventional activated sludge process includes (i) aerobic oxidation of biodegradable organic matter based on the conversion of soluble organic substances to CO_2, NH_4, H_2O, and synthesis of new cell biomass (activated sludge), and (ii) the separation of the treated wastewater and the activated sludge by sedimentation. Activated sludge is formed by a suspended-growth process of microorganisms followed by cell aggregate formation, the activated sludge flocs.

A conventional activated sludge WWTP includes an aeration tank and a sedimentation tank (clarifier). Primary effluent (wastewater after mechanical treatment) runs continuously into the aeration tank together with returned activated sludge in order to sustain a certain biomass concentration. The organic substances in the water phase are consumed by the biomass under completely mixed and aerated conditions in the aeration tank. Afterwards the suspended activated sludge enters the sedimentation tank where the biomass, present as flocs, settles due to gravity. A portion of the activated sludge in the clarifier is recycled back to the aeration tank. The remainder (excess sludge) is processed by different treatments such as thickening, dewatering, and stabilization (digestion). Afterwards sludge is disposed, e.g. to agricultural land, to landfill, or to incinerators. The purified water (secondary effluent) is released from the clarifier into open water bodies.

Nowadays further demands on wastewater treatment are stipulated because of eutrophication problems in rivers and lakes causes by high input of the nutrients nitrogen and phosphorus into surface waters followed by massive algal blooms. Besides degradation of carbonaceous substances, nutrient (nitrogen and phosphorus) elimination is required. Hence further purification steps were needed. After ammonification by proteolysis ammonia is oxidized to nitrate by nitrification. To eliminate nitrogen from the wastewater nitrate has to be reduced stepwise via nitrite and nitrous oxide to molecular nitrogen in the denitrification process. Phosphorus is usually eliminated chemically by precipitation. Nowadays a biological approach of enhanced biological phosphorus removal (EBPR) has been established in many WWTPs. These new purification steps require very different environmental conditions for optimal growth of the respective organisms. Nitrifying bacteria, as autotrophs, use CO_2 as a carbon source and are therefore most competitive under low load sludge. Denitrification takes place only under anoxic conditions with an organic carbon source being available and EBPR demands periodical limitation of oxygen in order to promote surplus P-uptake at aerobic conditions. The modern so-called nutrient removal plants are operated with anoxic and anaerobic zones in addition to aerobic environments. Furthermore the food to microorganism (F/M) ratio (sludge load) in the system needs to be partly low to support full nitrification (F/M ratio = 0.01-0.15 kg BOD_5 kg^{-1} MLSS d^{-1}).

2.2 Activated Sludge Flocs

Activated sludge flocs are known to be microbial aggregates embedded in a gel matrix. Extracellular polymeric substances (EPS) play an important role in the development of this specific matrix and represent a major sludge floc component beside water phase and cells (Li and Ganczarczyk 1990). In addition, inorganic and organic substances are adsorbed from the water phase to the flocs and hence sorption processes are involved in floc formation and consequently in the elimination process of wastewater pollutants. The structure and composition of activated sludge flocs are very complex and are directly or indirectly related to sludge settleability.

2.2.1 Microbial Biocenosis

Members of the domain *Bacteria*, which are the most effective degraders, represent the major organisms group within the activated sludge floc. They are responsible for oxidation of organic matter and nutrient transformation. Furthermore they produce extracellular polymeric substances, which support aggregation of microbial biomass. The different environmental conditions for carbon elimination and nutrient removal described above lead to the development of specific bacterial biocenoses characterized by a high diversity in municipal systems. Heterotrophic bacteria are involved in most of these processes. The majority of these bacteria belong to the *Alpha*- and *Betaproteobacteria* (Wagner et al. 1993, Manz et al. 1994, Snaidr et al. 1997). A great amount of research work has been done recently to obtain information about the autotrophic bacteria involved in nitrification (Burrell et al. 1998, Juretschko et al. 1998, Schramm et al. 1999), to characterize denitrifiers (Neef et al. 1996, Ginige et al. 2004), and to identify the active community in EBPR (Crocetti et al. 2000, Crocetti et al. 2002).

The ability of bacteria to build up filaments by incomplete cell division is a well-documented process. In activated sludge systems filamentous bacteria were often observed and it was suggested that they provide a stabilizing backbone for the three-dimensional floc structure (Bossier and Verstraete 1996). However, extensive growth of filamentous bacteria is often associated with settling problems such as bulking or scum formation. Bulking sludge is characterized by sludge flocs from which filamentous bacteria grow into the surrounding liquid inhibiting formation of dense floc aggregates under low hydrodynamic shear (e.g. during sedimentation). In contrast, scum formation is caused by sludge flocs float to the surface aggregating to a more or less stable sludge layer at the water-air interface (see chapter 2.3). According to Lemmer and Lind (2000) three different groups of filamentous bacteria involved in settling problems are frequently found in municipal WWTPs. Sulfur bacteria such as type 021N and *Thiothrix* sp., which are able to use beside organic substrates reduced sulfur components as energy source, and heterotrophic bacteria adapted to high sludge load (F/M ratio > 0.15 kg BOD_5 kg^{-1} MLSS d^{-1}), e.g. *Sphaerotilus* spp. and *Haliscomenobacter hydrossis*, are in general responsible for bulking sludge. The third group including heterotrophic bacteria adapted to low sludge load (F/M ratio < 0.15 kg BOD_5 kg^{-1} MLSS d^{-1}) is often found in nutrient removal plants with nitrogen elimination. Here both non-filamentous and filamentous bacteria are in general involved in scum problems with the latter partly causing bulking sludge (for details see 2.3.2).

Eukaryotic organisms are also found in activated sludge systems. However, activated sludge does not usually favor growth of fungi because of fungi being selected by extremely low pH values below 4. In contrast, monocellular protozoa comprising flagellates, amoeba, and ciliates, and highly organized metazoa such as rotifers, nematodes, and other worms play an important role in the activated sludge system. The primary role of both protozoa and metazoa is to clarify the effluent by predation on freely suspended bacteria and bacteria loosely attached to the floc surface.

2.2.2 Extracellular Polymeric Substances (EPS)

Various meanings and definitions of the term EPS are reported in the literature. The abbreviation EPS has been used for exopolymers, exopolysaccharides, extracellular polysaccharides, and extracellular polymeric substances. In one of the first reviews EPS was defined as "extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates" (Geesey 1982). Another definition for EPS can be found in the glossary to the report of the Dahlem Workshop on Structure and Function of Biofilms in Berlin 1989 (Characklis and Wilderer 1989): "EPS are organic polymers of microbial origin which in biofilm systems are frequently responsible for binding cells and other particulate materials together (cohesion) and to the substratum (adhesion)". Such biopolymers are synthesized and excreted by bacterial metabolism, and in addition originate from cell lysis. Data from pure cultures support the observation that many bacteria produce a range of EPS (Brown and Lester 1980, Jahn and Nielsen 1995). In more complex systems, e.g. the activated sludge floc, EPS originate from (i) microbial metabolism or lysis of microorganisms as described above and (ii) from wastewater components accumulated to the floc matrix by sorption processes (Urbain et al. 1993). In addition, hydrolysis processes of macromolecules due to the activity of extracellular enzymes, influence EPS composition (Frølund et al. 1995, Confer and Bruce 1998). Because it is not possible to distinguish between microbially produced EPS, adsorbed material, and hydrolysis products, in this work all three fractions are defined as EPS. Furthermore the terms "bound EPS" and "soluble EPS" are used for some biofilm systems (Hsieh et al. 1994, Nielsen et al. 1997). Bound EPS include sheaths, capsular polymers and cell-attached organic material. Soluble macromolecules, colloids, and slimes represent soluble EPS. This means that all polymers outside the cell wall, which are not directly bound to the outer membrane/murein-protein-layer, will be considered extracellular EPS material.

The composition of activated sludge EPS is very heterogeneous depending on the origin of the wastewater and the wastewater treatment process (Urbain et al. 1993, Frølund et al. 1994, Bura et al. 1998). Furthermore the extracted amount and relative fraction of the individual compound depends on the extraction methods used (Nielsen and Jahn 1999). The main organic fractions detected in activated sludge EPS were proteins, carbohydrates, uronic acids, humic substances, lipids, and fatty acids (Goodwin and Forster 1985, Urbain et al. 1993, Frølund et al. 1996, Bura et al. 1998, Dignac et al. 1998, Conrad et al. 2003, Wilen et al. 2003). Significant amounts of DNA and RNA were also found (Frølund et al. 1996, Palmgren and Nielsen 1996). Usually protein is determined as the predominant component with a protein to carbohydrate ratio of 2 to 8. The presence of large quantities of exoenzymes in the activated sludge floc (Frølund et al. 1995), required for the degradation of complex substrate present in wastewater, could explain the predominance of proteins in activated sludge EPS.

Extracellular proteins might also originate from both wastewater, which also contains a high amount of proteins (Raunkjær et al. 1994), and from cell lysis. Polysaccharides are mainly produced by the microbial biocenosis but might also be adsorbed from wastewater to the activated sludge floc and are enriched in the EPS after cell lysis. Humic substances represent the second main fraction in EPS and are supposed to originate from wastewater. Uronic acids, primary units of Gram-positive bacterial cell walls, and nucleic acids are found generally in low concentrations in activated sludge EPS and originate mainly from cell lysis. The lipid fraction within the sludge EPS was also determined in low concentrations of approximately 1-2% of the total sludge EPS. Three different sources were discussed as the origin of these lipids detected: (i) direct sorption of lipids and fatty acids (Quéméneur and Marty 1994, Hwu et al. 1998) and other wastewater compounds (Guellil et al. 2001) by activated sludge, (ii) cell lysis and (iii) metabolism of microorganisms (Finnerty 1988). With regard to the hydrophobic fraction of the EPS a high proportion of at least 7% of DOC was found by Jorand et al. (1998) in activated sludge flocs. This hydrophobic fraction consists mainly of proteins with no carbohydrates being determined.

The chemical composition of EPS is responsible for cohesion and mechanical stability of activated sludge flocs, which is important for floc aggregation under low hydrodynamic shear (flocculation) and consequently sludge settleability. Cohesion between the individual EPS molecules and additionally to cell surfaces is based on weak physicochemical interactions and not on covalent bonds. Repulsive or attractive electrostatical interactions and attractive van der Waals forces, described by the theory of Derjaugin, Landau, Verwey, and Overbeek (DLVO theory) for colloidal stability, and in addition hydrogen bonds were reported to be the major forces (Zita and Hermansson 1994, Mayer et al. 1999). Relatively weak individual binding forces as compared to a covalent C-C bond are developed by these interactions. However, EPS and cell surface provide large numbers of binding sites and hence total binding energies exceed those of covalent C-C bonds. Between hydrophobic substances, e.g. specific proteins composed of aromatic amino acids and lipids, van der Waals forces can be effective. In addition, hydrophobic interaction between these hydrophobic substances and the hydrophobic components of cell surfaces cause a stronger attraction of the EPS to the cell. Zita and Hermansson (1997a) showed a strong correlation between the hydrophobicity of cells and their degree of attachment to activated sludge. Bos et al. (1999) discussed that the attachment of hydrophobic cells to EPS is more based on the acid-base nature of the cell surface and not so much on the ability of the cell surface to exert van der Waals forces. Because of anionic functional groups in the EPS, e.g. phosphoryl, carboxyl, or sulfate groups, and cationic functional groups, e.g. amino groups, electrostatic interactions exist between the different polymers and the mainly negatively charged cell surfaces (polymer bridging model, Eriksson and Alm 1993, Urbain et al. 1993). Furthermore bivalent calcium acts as an important bridging ion between these described negatively charged sites on surfaces and polymers, which increase the stability of the network (Bruus et al. 1992, Keiding and Nielsen 1997). Additionally, groups with a high hydrogen bonding potential, such as polysaccharides, stabilize the EPS matrix. The different interactions described result in the formation of a three dimensional, gel-like network of EPS, which may vary dynamically in response to changes of environmental conditions.

A great amount of research work focused on the clarification of the functional role of EPS in flocculation process and sludge settleability. It was shown that surface interactions, which

positively influence sludge settleability, were directly related to a high content of polysaccharides and hydrophobic substances, e.g. lipids and proteins, within the EPS (Goodwin and Forster 1985, Higgins and Novak 1997). In contrast to these findings, other authors (Urbain et al. 1993, Liao et al. 2001) demonstrated that the relationship was inverse. Furthermore Urbain et al. (1993) found that at low EPS concentrations and when the internal hydrophobicity is high, settleability was improved. This indicates the involvement of internal hydrophobic bonds in flocculation mechanisms and their balance with hydrophilic bonds determines the sludge settling properties. The predominance of protein in the EPS, their high content of negatively charged amino acids involved in electrostatic forces and their hydrophobic components point out their key role in floc structure (Dignac et al. 1998). Wilen et al. (2003) demonstrated that EPS proteins had a relatively strong positive correlation with negative surface charge and flocculation ability. They concluded that protein had the biggest influence on surface properties and flocculating ability of the sludge flocs. Furthermore a significant correlation was observed between flocculation ability and hydrophobic properties of sludge flocs. Surface properties, such as hydrophobicity and surface charge, and EPS composition rather than the EPS quantity affect bioflocculation (Keiding and Nielsen 1997, Liao et al. 2001). With increasing ionic strength a higher floc stability was determined confirming the DLVO theory explained above (Zita and Hermansson 1994). In contrast, the EPS content is more important in controlling the settleability of sludge.

Additional possible functions of EPS are summarized by Wingender et al. (1999). EPS might act as protective barriers against toxic substances, e.g. heavy metals or certain biocides (disinfectants and antibiotics), predation and dramatic environmental fluctuations (pH, salt content, hydraulic pressure). Furthermore the localization of extracellular enzymes mentioned above, which perform the degradation of exogenous macromolecules and particulate material, is well described in the literature. This observation indicates two further functional aspects, which are described in the following chapters, the involvement of EPS in accumulation and subsequent utilization of these accumulated substances as carbon sources.

2.2.3 Sorption Process

Besides microbial degradation, sorption processes of organic and inorganic substances to the activated sludge flocs affect the pollutant elimination in activated sludge wastewater treatment. Two different sorption phenomena can be differentiated, the attachment of the substance to the surface of the activated sludge floc (adsorption) and the accumulation of the substances inside the flocs. In general an equilibrium between both sorption processes is developed (Weber et al. 1991). The term biosorption includes the sorption processes of metals, organic substances, and particulate material to biological material, i.e. activated sludge floc.

The mechanisms involved in the sorption of organic and inorganic substances, and in addition the attachment of bacterial cells to sludge flocs, are the same as described above for cohesion including electrostatic forces, van der Waals interactions, Lewis acid-base forces and hydrogen bonding. Flemming (1995) described different sorption sites such as EPS, cell wall, cell membranes, and cell cytoplasm in biofilms, which are also found in activated sludge flocs. EPS display differently charged groups, nonpolar groups, and groups with hydrogen bonding potential mentioned above. The cell wall consists of N-acetylglucosamine and N-

acetylmuramic acid providing cationic and anionc sites. The cell wall of nocardioform actinomycetes is rich in covalently bound hydrophobic mycolic acids with a long aliphatic chain consisting of 30 up to 90 carbon units, which are exposed to the environment (Neu 1996) and might act as sorption site for hydrophobic compounds. The cytoplasmic membrane and the outer membrane of Gram-negative cells are mainly composed of phospholipids, which represent the most probable site of lipophilic substances. The outer layer of the outer membrane of Gram-negative cells contains amphiphilic lipopolysaccharides anchored with the hydrophobic or hydrophilic part in the outer layer of the cell surfaces. Amphiphilic lipoteichoic acids are bound to the cell wall of Gram-positive cells. Therefore both lipopolysaccharides and lipoteichoic acids might act as sorption sites for hydrophobic or hydrophilic substances. A further potential site for binding and accumulation of dissolved substances is the interior of the bacterial cell, the cytoplasm, where these compounds might be stored as reserve material. The description of the various structures within the activated sludge characterized by charged and nonpolar functional groups indicates that different sorption preferences, capacities and properties exist for water, inorganic solved substances, organic solved substances, and particulate material.

The sorption processes in activated sludge systems were investigated by different research groups. Guellil et al. (2001) determined a biosorption capacity of around 40 to 100 mg COD g^{-1} TSS, consisting on average of 45% of the non-settleable (i. e. colloidal and soluble) fraction of the wastewater from a municipal WWTP. The transfer of soluble fraction and colloidal matter reached a steady state after 40 min and 20 min, respectively. It was assumed that the steady state obtained for soluble substances is delayed because of its diffusion into the floc matrix, whereas colloids do not penetrate into the matrix because of their size. The investigation of the sorption process of specific substances such as long-chain fatty acids revealed that the initial adsorption rate takes approximately one day but sorption equilibrium required much more time (weeks, Hwu et al. 1998). Furthermore they showed that sorption of long-chain fatty acids is a prerequisite for their biodegradation. A significant removal of hydrophobic pollutants, such as halogenated hydrocarbons (40%) and polycyclic aromatic hydrocarbons (75%), by sorption to activated sludge flocs is reported by Finlayson et al. (1998). The high sorption rate of these hydrophobic substances had no significant effects on the floc structure and sludge settling. With regard to the sorption site it was demonstrated by Späth et al. (1998) in a biofilm system that more than 60% of hydrophobic organic pollutants (benzene, toluene and xylene) were found in the EPS, whereas more than 80% of the total content of heavy metals were localized in the cellular fraction. The sorption capacity of organic substances to activated sludge increased with the hydrophobicity of the substances as was shown for different phthalates (Fang and Zheng 2004). Furthermore pH, calcium content, and ionic strength influence biosorption processes. Higher biosorption of high molecular weight humic substances to activated sludge was determined at pH 6, increasing calcium concentration, and ionic strength (Esparza-Soto and Westerhoff 2003). The increasing biosorption at low pH might be attributed to hydrophobic interactions between humic substances and activated sludge. Desorption of organic macromolecules from activated sludge was observed after the removal of calcium ions by an ion exchange resin indicating that the ionic composition plays a very important role in the sorption process of organic material to activated sludge flocs (Keiding and Nielsen 1997). Ionic strength influences as well bacterial adhesion to the activated sludge flocs (Zita and Hermansson 1994). In addition, cell surface

hydrophobicity is a very important factor influencing the relative bacterial adhesion potential (Zita and Hermansson 1997a, b, Olofsson et al. 1998). The major fraction of free-living cells had hydrophilic cell surfaces and in addition, it was observed that hydrophilic cells attached only to the floc surface. In contrast, hydrophobic bacteria penetrate the flocs through channels and pores. Furthermore surface tension might play an important role in bacterial adhesion processes and consequently in floc aggregation. Olofsson et al. (1998) demonstrated that a decrease in surface tension reduced adhesion of bacterial cells indicating pollution by various surfactants might to have a negative effect on aggregation.

2.2.4 EPS as Nutrient Source

The role of EPS in nutrient supply is still not well understood. Little is known about the extent to which nutrients in bacterially produced EPS can be utilized, either by the same organisms, or by cross-feeding between species (Wolfaardt et al. 1999). It has been suggested that EPS may be produced by some bacteria as a nutrient reserve for long-term storage of carbon and energy. Kuehn et al. (2001) observed in monoculture studies on a *Sphingomonas* strain EPS reduction during biofilm development. Therefore bacterial cells might be able to break down their own EPS and use the resulting degradation products as carbon and energy source. In addition, some bacteria could utilize the polymers of other bacteria providing an opportunity of cross-feeding. A second important aspect might be the fact that the EPS act as a nutrient trap. Wolfaardt et al. (1995) showed in a multispecies biofilm community the accumulation of a specific herbicide within the EPS and subsequent degradation of adsorbed herbicide in the absence of another carbon source. The aggregation of a diverse microbial biocenosis leads to synergistic microconsortia, which facilitate the sequential degradation of substances being not readily biodegradable by single species populations. A recent study (Zhang and Bishop 2003) discovered that EPS are biodegradable by their own producers and by other microorganisms under starvation conditions with EPS carbohydrates being utilized faster than EPS proteins.

The transformation of lipids in activated sludge was studied by Dueholm et al. (2001). They suggested that the key steps are the adsorption of lipids, such as long-chain fatty acids and triglycerides, to sludge flocs, the hydrolysis of triglycerides by lipases, and the uptake of long-chain fatty acids by bacteria. The sorption of organic substances described in chapter 2.2.3 leads to an accumulation of potential nutrients within the activated sludge floc. Extracellular enzymatic activity in the activated sludge floc matrix was described by different authors (Nybroe et al. 1992, Frølund et al. 1995, Schade and Lemmer 2005). The hydrolysis of exogenous macromolecules by these extracellular enzymes provides low-molecular-weight nutrients, which can readily be taken up and metabolized by cells, corroborating the role of activated sludge EPS as nutrient source.

2.3 Scum Formation

Scum is defined as a stable, brownish and viscous biomass layer between 10 cm and 1.5 m deep floating on the water surface of e.g. aeration tank (Fig. 1) or secondary clarifier. Furthermore scum also appears in anaerobic digesters. Scum is mostly characterized by a 5 up to 20 time higher biomass concentration as compared to dispersed activated sludge. In the literature terms such as scum, foam, or floating sludge were often used synonymously to

describe this phenomenon. Scum is however different from the foams caused by non-biodegradable surfactants, which in general could be destroyed by water spraying.

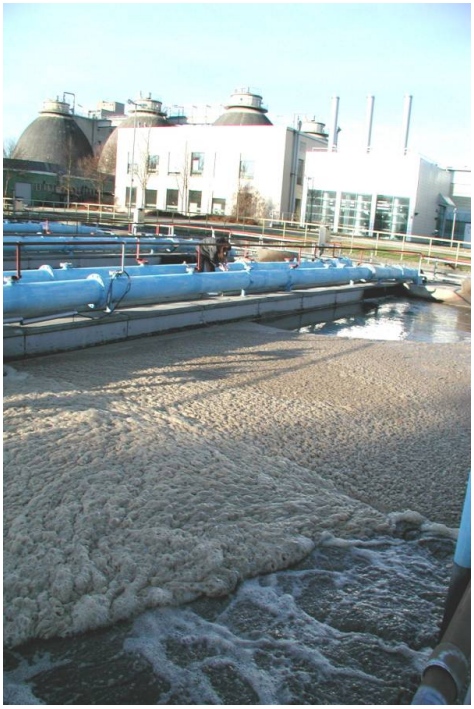


Figure 1: Stable scum layer floating on the aeration tank (C-elimination and pre-denitrification) of a municipal WWTP.

Scum formation is in addition to bulking sludge a widespread problem all over the world and appears most intense during winter and spring. Different reports describe this problem in many countries, including USA (Pitt and Jenkins 1990), Australia (Seviour et al. 1990, Blackall et al. 1991a, Seviour et al. 1994), Italy (Madoni et al. 2000), France (Pujol et al. 1991), Czech Republic (Wanner et al. 1998, Krhùtková et al. 2005), Germany (Lind and Lemmer 1998, Paris 2005), Great Britain (Goodfellow et al. 1996, de los Reyes et al. 1998) and South Africa (Blackbeard et al. 1986). Serious operating problems occur in the presence of thick scum layers, which are: (i) troubles in liquid/solid separation and consequently deterioration of the effluent quality and (ii) transport and dispersal of pathogens via wind blown scum. Further scum in anaerobic digesters might cause overflow and consequently blocking of gas pipes and relief systems.

2.3.1 Scum Development

Three factors are necessary for stable scum formation: (i) dispersed gas bubbles, (ii) surface-active compounds and (iii) hydrophobic material (Soddell and Seviour 1990). The source of the gas bubbles is variable. They are present in the process as air or oxygen in the aeration tank, nitrogen or nitrous oxide in the denitrification zone, or methane in the anaerobic digester. However, Lemmer and Baumann (1988b) found no evidence that nitrogen gas originating from the denitrification process might be involved in scum formation.

Gas bubbles in a liquid would collapse immediately. For stabilization of their gas-water interface surface-active molecules are necessary to adhere onto the interface that reduces

surface tension and initiates foam production. Persistent scum layers are achieved in the presence of hydrophobic material. The hydrophobic properties of the particles provide stable adhesion of gas bubbles to particles. This reduces the liquid content and promotes persistence of scum.

Surface-active materials may enter the WWTP via primary effluent or be produced by various bacteria under nutrient deficiency and/or the presence of hydrophobic carbon sources. Synthetic surfactants originating from washing powders and cleaning agents have been discussed as a causative agent for scum production. However, many modern synthetic surfactants are readily degradable and therefore no accumulation in the sludge fraction takes place (Schade and Lemmer 2001). The production of biosurfactans is reported for many organisms. The data obtained by Goddard and Forster (1987) indicated that scum formation might be associated with the production of specific biosurfactants. The nature of these surface-active agents appears to be a function of the species involved and the composition of the available nutrients. *Pseudomonas* spp., especially *P. aeruginosa,* are known to excrete large quantities of rhamnolipids, which stimulate growth on n-hexadecane. However, biosurfactants were produced as well during growth on non-hydrophobic substrates such as glucose (Rosenberg 1986, Lang 1999). The formation of rhamnolipids was greatly increased by nitrogen limitations. The production of emulsifying minicapsules on the cell surface containing emulsan, a polymeric biosurfactant (lipoheteropolysaccharide), was determined for *Acinetobacter calcoaceticus* growing on hydrocarbon as carbon source (Rosenberg 1986, Lang 1999). In pure culture studies it was observed that during late exponential and early stationary growth phases emulsan is released into the medium, an effect that is probably enhanced by starvation conditions. Nocardiofom actinomycetes, e.g. *Rhodococcus erythropolis,* respond to the presence of alkanes by producing glycolipids, e.g. trehalose lipids, which remain cell bound (Lang and Philp 1998).

Gochin and Solari (1983) indicated hydrophobic sites to be absolutely essential because bubbles entrapment into hydrophilic flocs would not be able to rise the particles to the water surface. Hydrophobic material such as oil and grease might originate from the primary effluent. In addition, hydrophobic particles seem also to be available by an abundant growth of organisms with hydrophobic cell surfaces. Blackall and Marshall (1989) demonstrated for nocardioform actinomycetes-borne scum that both biosurfactants and hydrophobic cell surfaces are necessary for stable scum formation. Cell surface hydrophobicity is promoted by cell-bound biosurfactants anchored with the hydrophilic part in the outer layer of the cell surface. Phospholipids, protein, and lipoprotein components of the inner layer of the outer membrane and lipopolysaccharides covalently attached to the outer layer of the outer membrane influence the hydrophobicity of Gram-negative cells. The very thick peptidoglycan layer and lipoteichoic acid bound to the peptidoglycan layer found in Gram-positive cell envelopes affects cell surface hydrophobicity of Gram-positive bacteria. It is known that cell surface hydrophobicity is influenced by the prevailing substrate and milieu conditions. Many microorganisms strongly increase their hydrophobicity by removing their polysaccharide slime capsule during starvation phases with carbonaceous substrate limitation (Kjelleberg and Hermansson 1984) and by biosurfactant production as described above. Furthermore it was shown that *Gordonia amarae* produces hydrophobic cell surface fimbriae during the growth on hydrocarbon (Cairns et al. 1982).

2.3.2 Microbial Populations associated with Scum

Microscopic examination of scum usually reveals large numbers of filamentous bacteria (Lind and Lemmer 1998) and even non-filamentous bacteria have been reported as constituting the predominant microbial populations in some scum samples (Seviour et al. 1990, Lemmer et al. 1998). Lind and Lemmer (1998) observed a distinct increase of WWTPs with scum events from 1989 to 1996. A recent questioning study performed from 2002 to 2003 demonstrated that 38% of the interrogated WWTPs (48 German WWTPs) suffered from scum (Paris 2005). A significant shift from Gram-negative filamentous and non-filamentous organisms to Gram-positive bacteria was simultaneously found during the last 15 years. This shift of bacterial populations correlates quite well with the change of operating conditions in the early 1990s. Nutrient removal plants at present perform nitrogen and phosphorus removal additionally to carbon elimination and require specific operating parameters described in chapter 2.1. Therefore nowadays organisms have to adapt to low loaded sludge and occasional anaerobic conditions. This favors the development of a specific microbial biocenosis that is characterized by a low growth rate and consequently demands a higher sludge age. Filamentous bacteria morphotypes such as *Microthrix parvicella*, nocardioform actinomycetes, *Nostocoida limicola*, and Eikelboom types 0041/0675, 1851, and 0092 turned out to be numerous in nutrient removal plants described. Most of these bacteria were discussed to be characterized by hydrophobic cell surfaces and the ability to produce biosurfactants. Both properties are required for the utilization of hydrophobic substrate and moreover support the development of stable scum layers.

In Table 1 a literature overview shows the frequency of these scum bacteria in WWTPs with scum events from different countries. *M. parvicella* is the most frequently detected filamentous bacterium in WWTPs with scum problems all over the world and is found at high numbers in both activated sludge and scum fractions. Nocardioform actinomycetes growing as Gram-positive branched filaments are described as the second important bacteria group involved in scum formation and are in general enriched in the scum layer. This group, characterized by two different morphotypes (GALO and PTLO, see chapter 2.3.2.2), represents the major organism group associated with scum formation in the US and in Eastern Australia. Filamentous bacteria types 0092, 1851, 0041/0675, and *N. limicola* were reported only occasionally to be involved in scum formation. Type 0092 seemed to be much more prominent in South Africa where it occurred as the dominant bacterium in the same percentage of WWTPs as did *M. parvicella.* But in contrast to *M. parvicella*, type 0092 was not accumulated selectively in the scum fraction and might be only incidentally trapped from activated sludge into the scum layer (Blackbeard et al. 1988). Therefore it was discussed that type 0092 might not be a true scum organism. Furthermore it is of interest that scum fractions that are associated with high numbers of *N. limicola* are less stable as compared to scum layers enriched with nocardioform actinomycetes or *M. parvicella* (Khan et al. 1991, Foot et al. 1993). In contrast, the combination of *N. limicola* and type 0041 produced stable scum in a laboratory scale experiment (Goddard and Forster 1987). Furthermore both *N. limicola* and type 0041 caused the first serious case of activated sludge scum formation in a Czech WWTP (Wanner and Grau 1989). In the scum fraction *N. limicola* and type 0041 predominated in a 1:2 ratio, whereas in activated sludge the same dominant filamentous organisms appeared in the converse ratio.

Table 1: Dominant filamentous scum bacteria in WWTPs with scum events from different countries (identification according to their morphology, Gram- and Neisser-staining features).

country	ranking			references
	1	2	3	
Czech Republic	*M. parvicella*	*N. limicola* type 0092 nocardioform actinomycetes (GALO[a])	type 1851 type 0041/0675	(Wanner et al. 1998, Krhùtková et al. 2005)
France	*M. parvicella*	type 0041/0675	nocardioform actinomycetes (GALO)	(Pujol et al. 1991, Duchene 1994)
Germany	*M. parvicella*	nocardioform actinomycetes (GALO) type 0041/0675	type 1851 type 0092 *N. limicola*	(Lind and Lemmer 1998)
Great Britain	nocardioform actinomycetes, *M. parvicella*	nocardioform actinomycetes	*N. limicola*	(Kerley and Forster 1995, Goodfellow et al. 1996)
Italy	*M. parvicella*	nocardioform actinomycetes (GALO)	type 0041/0675	(Madoni et al. 2000)
the Netherlands	*M. parvicella*	nocardioform actinomycetes	*N. limicola*	(Eikelboom 1994)
Sweden	*M. parvicella*	no report	no report	(Dillner Westlund et al. 1996)
USA	nocardioform actinomycetes (GALO) *Gordonia* spp. *M. parvicella*	*M. parvicella* nocardioform actinomycetes (PTLO[b])	no report	(Pitt and Jenkins 1990, de los Reyes et al. 2002)
Australia Victoria	*M. parvicella* nocardioform actinomycetes (GALO)	nocardioform actinomyctes (PTLO)	type 0092	(Seviour et al. 1990)
Australia New South Wales	*M. parvicella*	nocardioform actinomyctes (GALO and PTLO)	type 0092 type 0041/0675 type 1851 *N. limicola*	(Seviour et al. 1990)
Australia Queensland	nocardioform actinomycetes (GALO)	*M. parvicella* nocardioform actinomycetes (PTLO)	type 0092 type 0041/0675 type 1851 *N. limicola*	(Seviour et al. 1990, Blackall et al. 1991a)
South Africa	type 0092 *M. parvicella*	nocardioform actinomycetes	type 0041/0675 type 0803 type 0914	(Blackbeard et al. 1986)

[a] GALO = *Gordonia amarae*-like organisms
[b] PTLO = Pine tree-like organisms

Most of these filamentous scum bacteria exhibit similar environmental requirements such as nutrient, temperature, and oxygen demand. Therefore some of these bacteria were found together competing for the same nutrients but with *M. parvicella* or nocardioform actinomycetes in general to predominate. However, also distinct differences in milieu and substrate requirements were observed. Furthermore a shift of scum bacteria correlated to seasonal conditions or operating parameters was determined, e.g. *M. parvicella* to proliferate during winter/spring and type 0092 during summer (Eikelboom et al. 1998). In the following chapters literature data available for the taxonomic classification of the described scum bacteria and their morphological and physiological properties are reported. This information might provide a better understanding for the development of these scum bacteria and indicates their possible role in scum formation, which is the basis for specific scum control strategies.

2.3.2.1 *Microthrix parvicella*

The first detailed description of the Gram-positive filamentous bacterium *Microthrix parvicella* found in bulking sludge was given by van Veen (1973), who proposed the name *Microthrix parvicella* based on morphological data. According to the identification key system developed by Eikelboom (1975, 2000) *M. parvicella* forms thin Gram-positive, coiled, non-branched filaments, with about 0.3 to 0.6 µm diameter and 300 to 500 µm length. Electron microscopic analysis showed that the cells contain electron-dense as well as transparent granules. The electron-dense intracellular granules consist of polyphosphate, which was confirmed by a positive Neisser reaction. Some kind of reserve material is presumably stored in the electron-transparent granules.

For lack of a better approach researchers have attempted to isolate and cultivate *M. parvicella* for around 30 years. However, it turned out growing *M. parvicella* in pure culture is very difficult. Both van Veen (1973) and Eikelboom (1975) successfully isolated several pure cultures identified as *M. parvicella* and published several morphological data presented above. The work of Slijkhuis (1983a, 1983b, Slijkhuis et al. 1984, Slijkhuis and Deinema 1988) focused on detailed physiological characterization of *M. parvicella*. The studies identified long-chain fatty acids (LCFAs) and their Tween esters to be the only carbon sources utilized by this microorganism. Volatile fatty acids (VFAs), such as acetate and butyrate, and medium length fatty acids support the growth only in the presence of LCFAs. Years later these observations were confirmed by in situ studies with activated sludge (Andreasen and Nielsen 1997, 1998, 2000). The application of microautoradiography showed that *M. parvicella* did not take up simple organic substrates such as acetate, ethanol, and glucose, and was not able to utilize the detergent sodium dodecyl sulfate (SDS) and a long chain fatty alcohol (octadecanol) either. Among the substrates tested only LCFAs, oleic acid, and palmitic acid, and to some extent their triglycerides were taken up as carbon sources by *M. parvicella*. The lipid triglyceride was hydrolyzed in the investigated sludge and *M. parvicella* was able to take up the released LCFAs. In contrast to Slijkhuis (1983a), who determined *M. parvicella* as strictly aerobic bacterium, Andreasen and Nielsen (1998, 2000) found out oleic acid to be taken up under anaerobic, anoxic, and aerobic conditions. *M. parvicella* has a high storage capacity of LCFAs as neutral lipids under aerobic (Slijkhuis 1983a), anoxic, and anaerobic conditions, and metabolizes them under aerobic and anoxic conditions (Andreasen and Nielsen 2000, Nielsen et al. 2002, Hesselsoe et al. 2005). These esters are presumed to be the main component of large intracellular lipid granules detected

microscopically (Slijkhuis et al. 1984, Slijkhuis and Deinema 1988). LCFAs might act as sole carbon source for *M. parvicella*, however, Slijkhuis (1983b) pointed out LCFAs to be toxic at concentrations above 0.025 mg oleic acid per mg dry matter. In contrast, Tween 80 does not show a similar inhibitory effect. On the other hand, nocardioform actinomycete species tolerate two orders of magnitude higher LCFA concentrations. Nevertheless *M. parvicella* seems to be a specialized lipid consumer and shows specific cell surface properties. Its relatively hydrophobic cell surface eases LCFA substrate uptake. Extracellular lipases on the cell surface allow the degradation and consumption of lipids (Nielsen et al. 2002, Schade and Lemmer 2005).

Paris (2005) investigated in a pilot plant experiment, where good growth of *M. parvicella* was observed on municipal wastewater supplied with vegetable oil and/or Tween 80, the control mechanism of aluminum chloride. The dosage of aluminum chloride (3.5 mg Al^{3+} g^{-1} MLSS d^{-1}) improved sludge settleability accompanied with a decrease of hydrophobic and floating sludge properties. Simultaneously a change of *M. parvicella* morphology was determined, growing as short filament inside the flocs. Schade and Lemmer (2005) detected in the same experiment lipase activity of *M. parvicella* to be decreased after the dosage of 2.2 mg Al^{3+} g^{-1} MLSS d^{-1} and to be increased gradually after stopping of aluminium dosage. Hence Paris (2005) concluded that aluminum chloride cause co-precipitation of lipids and by this the carbon source for *M. parvicella* was taken away, which causes reduction of *M. parvicella* growth. A further effect of aluminum chloride was a decline of activated sludge hydrophobicity by inclusion of the hydrophobic sludge sides by aluminum compounds. Nielsen et al. (2005) demonstrated on batch-scale experiments with poly aluminum salts (PAX-14, 10 to 15 mg Al^{3+} g^{-1} MLSS) a reduction in substrate uptake of LCFAs by *M. parvicella* and a decline of lipase activity. No alternation in the cell surface hydrophobicity was observed, however, the flocculating effect of PAX-14 seemed to improve the development of dense flocs embedding *M. parvicella* inside the floc material.

The growth rate of *M. parvicella* in pure culture is quite low and generation times of 12 to 43 h were determined (Slijkhuis 1983a). This observation explains the occurrence of this organism in WWTPs that are operated at high sludge age (> 20 d) and under low sludge load conditions with an F/M ratio of 0.05 to 0.2 kg BOD_5 kg^{-1} MLSS d^{-1} (in Lemmer and Lind 2000, p. 60). Furthermore optimal growth of *M. parvicella* was achieved at temperatures below 20°C (Slijkhuis 1983b, Knoop and Kunst 1998, Lind and Lemmer 1998) and in the presence of reduced nitrogen compounds, especially ammonium, and sulfur sources (Slijkhuis 1983b). *M. parvicella* needs a pH over 7 (Slijkhuis and Deinema 1982) and hence *M. parvicella* is in general not present in nitrification tanks where lower pH values are measured. The development of a *M. parvicella* population seems to be stimulated rather by aerobic (C-elimination, nitrification), anoxic (denitrification) and anaerobic (EBPR) zones being performed in the same reactor than by process configurations based on separated zones (Eikelboom et al. 1998). This indicates *M. parvicella* to be more competitive under dynamic aerobic and anaerobic conditions. This is probably due to its storage capacity of LCFAs in an anaerobic environment as mentioned above, where only few other bacteria are able to take up substrate (Nielsen et al. 2002). The substantial carbon substrate uptake under anaerobic conditions was not accompanied by a fast phosphate uptake and storage under subsequent anoxic and aerobic conditions as described for phosphorus accumulating organisms (PAO) (Andreasen and Nielsen 2000). In contrast to these observations, the positive response to

Neisser staining indicates polyphosphate to be accumulated in granules inside the cells (Slijkhuis 1983a, Eikelboom 2000).

The 16S rDNA comparative analysis was applied to clarify the phylogenetic position of *M. parvicella*. Blackall and co-workers (1994b, 1996b) found that isolates identified morphologically as *M. parvicella* represent a novel deep branching member of the *Actinobacteria* phylum. The 16S rDNA sequences of two clones from a 16S rDNA clone library originating from Australian activated sludge enriched with large numbers of *M. parvicella* (Blackall et al. 1996b) and of one Italian *M. parvicella* isolate (Rossetti et al. 1997a) were phylogenetically similar to the original Australian isolates. *M. parvicella* was most closely related to *Acidomicrobium ferrooxidans* (formerly an unnamed facultatively thermophilic, iron-oxidizing strain TH3, 87% sequence similarity), to *Arthrobacter globiformis* (84.5%) and to *Nocardioides simplex* (84%) (Blackall et al. 1994b). The study of Blackall et al. (1994b) showed that the average similarity of *M. parvicella* within the order *Actinomycetales* (including the nocardioform actinomycete group) was 83%, within the genus *Atopobium* similarity it was 81% and within the *Firmicutes* phylum it was 79%. Because of these low sequence similarities it was suggested that the *Microthrix* group constitutes a novel order within the *Actinobacteria* phylum. However, more strains are required to verify this hypothesis and hence *M. parvicella* is described as an unclassified member of the *Actinobacteria*. There are still difficulties to keep these organisms in culture and consequently at present not enough phenotypic and chemotaxonomic data exist for any *M. parvicella* strain to validly name the organism. Blackall et al (1996b) proposed the name "*Candidatus* Microthrix parvicella". The category *Candidatus* is recommended to describe uncultivated prokaryotes, whose uniqueness is defined only by very limited characteristics such as 16S rDNA sequences (Murray and Schleifer 1994, Murray and Stackebrandt 1995). For "*Candidatus* M. parvicella" strain approximately 66% of the 23S rDNA were determined (Bradford et al. 1998). The results demonstrated that the probe HGC69a targeting a specific region of the 23S rRNA of the *Actinobacteria* varied in at least two nucleotides with the 23S sequence of "*Candidatus* M. parvicella". Furthermore an approximately 100 nucleotide stable insert between helices 54 and 55 in the 23S rDNA specific for the *Actinobacteria* phylum was detected in "*Candidatus* M. parvicella" as well. However, the length (79 nucleotides) and sequence of this insert was unique for "*Candidatus* M. parvicella". Erhart et al. (1997) designed four different 16S rRNA targeted oligonucleotide probes, detecting different sites of the 16S rDNA of "*Candidatus* M. parvicella". FISH technique with these probes was applied to gain information about the global diversity of *M. parvicella*. It could be determined that the morphotypes *M. parvicella* found in different WWTPs of Germany, Italy, and Australia were the same organism. However, more data are necessary to conclude whether the morphotype *M. parvicella* growing in WWTPs all over the world represents one phylogenetic entity.

2.3.2.2 Nocardioform Actinomycetes

The first reports relate scum events to an abundant growth of nocardioform actinomycetes (Lechevalier and Lechevalier 1974, Pipes and Wesley 1978). In these early days mainly *Nocardia amarae* – meanwhile being reclassified to *Gordonia amarae* – that develops Gram-positive branched filaments at right angles has been postulated to be associated with scum formation. Therefore these organisms were referred to as *Nocardia amarae*-like organisms (NALO) or - because of the reclassification of *N. amarae* to *G. amarae* - to *Gordonia*

amarae-like organisms (GALO). However, Lemmer and Kroppenstedt (1984) isolated a variety of nocardioform actinomycetes belonging to the *Rhodococcus rhodochrous* group, the *Tsukamurella paurometabolum* group (formerly *Gordonia aurantiaca*), and *Gordonia amarae*. These organisms develop Gram-positive branched filaments as described above. In addition, *Skermania piniformis* was revealed as an important scum bacterium (Blackall et al. 1989, Chun et al. 1997). This organism develops Gram-positive filaments with acute angle branching similar to the appearance of a pine tree and for that reason this morphotype is called Pine tree-like organisms (PTLO). The PTLO morphotype has traditionally been attributed to *Skermania piniformis* whereas the GALO morphotype belongs to many nocardioform genera. However, Soddell and Seviour (1998) observed that some filaments exhibit both acute- and right-angled branching but without the characteristic tree shape. Probably they represent new species. Recent phylogenetic and phenotypic studies of nocardioform actinomyctes present in WWTPs revealed the high diversity amongst this group and it was shown that different genera such as *Dietzia, Gordonia*, *Mycobacterium*, *Rhodococcus*, *Tsukamurella*, and *Skermania piniformis* are involved in scum formation (Schuppler et al. 1995, Goodfellow et al. 1998, Soddell et al. 1998, Seong et al. 1999, Stainsby et al. 2002, Wagner and Cloete 2002, Nam et al. 2003). Because of the described wide diversity amongst the nocardioform filaments associated with scum and the fact that according to the new taxonomic classification no *Nocardia* spp. are involved, the term NALO is usually used in the context of "NocArdioform-Like Organisms".

According to the proposal of Stackebrand et al. (1997) the nocardioform actinomycete group as well known as mycolata is classified as a new suborder *Corynebacterineae* within the order *Actinomycetales* affiliated with the class *Actinobacteria.* This suborder contains the families *Nocardiaceae* including *Nocardia* spp. and *Rhodococcus* spp., *Gordoniaceae*, *Mycobacteriaceae*, *Dietziaceae*, *Tsukamurellaceae* and *Corynebacteriaceae*. The organism *Skermania piniformis* is not listed in this proposal. However, Chun et al. (1997) reported that *Skermania piniformis* represents a distinct phylogenetic line that is most closely related with the *Gordonia* clade. The taxonomic outline of the prokaryotic genera in Bergeys Manual of Systematic Bacteriology (Garrity et al. 2004) classified *Skermania piniformis* within the family *Gordoniaceae*. Further, the family *Williamsiaceae* is affiliated with the suborder *Corynebacterineae*. Nocardioform actinomycetes have in common the described mycelial growth (“nocardioform”), the characteristic cell wall type IV with the taxonomically significant constituents meso-diaminopimelic acid, arabinose, and galactose, and the production of hydrophobic mycolic acids (mycolata) present with genus-dependent carbon lengths from 30 to 90 (Lechevalier 1989). A similar branching morphology is found in most of the genera within the nocardioform actinomycete group. In addition, non-filamentous growth is found as well and the development of specific morphotypes is often dependent on environmental conditions or growth phase. Lemmer (1985) determined typical branched filaments in the exponential stage that disintegrated into single rods or coccoid cells during the stationary growth phase. An identification by morphology criteria only is consequently unsound. The development of 16S rRNA targeted oligonucleotide probes for the group nocardioform actinomycetes (Schuppler et al. 1998, Davenport et al. 2000), for *Gordonia* (de los Reyes et al. 1997) and for *Skermania piniformis* (Bradford 1997) makes it possible to identify these organisms in situ and independently of morphology. However, so far there is

still a significant deficit in probes to characterize most of the nocardioform actinomycetes at genus and species level.

Optimal growth conditions of nocardioform actinomycetes vary between the various species. The temperature optima show strong differences but in general most of them tolerate relatively high temperatures. *Gordonia amarae* strains isolated (i) from a paper mill WWTP with an average wastewater temperature of 30°C (Lemmer 1985) and (ii) from Australian municipal WWTPs with generally high wastewater temperatures (Soddell and Seviour 1995) grew between 25 to 30°C with an optimum growth at 28°C. For *Skermania piniformis* a similar temperature range from 15 to 31°C was found with optimal growth at 18 to 25°C (Blackall et al. 1989). In contrast, *Rhodococcus* spp. and *Tsukamurella* spp., originating from German and Swiss municipal WWTPs with average temperatures in the aeration tank of 9°C in winter and 16°C in summer, prefer lower temperatures in the range from 8 to 25°C (Lemmer 1985). A higher temperature range from 5 to 40°C was determined for different *Rhodococcus* spp. isolated by Soddell and Seviour (1995). However, the authors suggested that nocardioform actinomycetes associated with scum at low temperature are more likely belonging to *Rhodococcus* species. *Gordonia* spp. and *Skermania piniformis* were in general found more frequently in countries characterized by a warmer climate, e.g. Australia, or industrial WWTPs, whereas *Rhodococcus* spp. and *Tsukamurella* spp. were present in higher numbers in regions with moderate climate (Soddell et al. 1992). The pH-tolerance as well differs between these groups, *Rhodococcus* spp. and *Tsukamurella* spp. showed an optimal growth rate between pH 5 and 9. In contrast, *Gordonia amarae* needs a pH range from 5 to 7 (Lemmer 1985). Nocardioform actinomycetes detected in scum were described as aerobic organisms. In anoxic zones they were able to reduce nitrate to nitrite, however, uptake of carbon sources seemed to be possible under aerobic conditions only (Lemmer 1985, Blackall et al. 1991c, Goodfellow et al. 1996). In contrast, recent in situ studies applying microautoradiography showed for *Skermania piniformis* in scum environments that substrate uptake was possible under both aerobic and anaerobic conditions, and to a small extent in an anoxic environment (Eales et al. 2005). Nocardioform actinomycetes are able to use nutrient components such as nitrogen, phosphorus, and sulfur either in reduced or oxidized form (Lemmer 1985). Polyhydroxybutyric acid (PHB) was detected as reserve material within the cells of nocardioform actinomycetes as investigated by Lemmer (1985). *Rhodococcus* strains such as *R. opacus* growing on n-alkanes and gluconate stored neutral lipids that consisted mainly of triacylglycerols instead of PHB or polyhydroxyalkanoates (Alvarez et al. 1996). A further reserve component is polyphosphate stored in volutin granules. However, nocardioform actinomycetes are not representing typical PAO.

Nocardioform actinomycetes are able to use a variety of substrates. These substrates include readily degradable substances such as mono- and disaccharides, and volatile fatty acids. However, poorly degradable substances such as long-chain fatty acids (LCFAs), alkanes, polycyclic aromatic hydrocarbons, or polyhalogenated hydrocarbons might serve as carbon and energy source as well (Lemmer and Kroppenstedt 1984, Goodfellow et al. 1996, Soddell et al. 1998). In the study of Soddell et al. (1998) growth of different nocardioform actinomycete isolates on hydrophobic substrate is reported. *Gordonia* spp. (*Gordonia* sp. and *Gordonia amarae*), *Rhodococcus* spp. (*Rhodococcus* sp. and *Rhodococcus erythropolis*) and *Skermania piniformis* were tested for growth on different oils, glycerol trioleate, kerosene, and hexadecane. *Rhodococcus erythropolis* showed optimal growth rates on all substrates

tested whereas none of the other strains was able to degrade hexadecane. All of them grew moderately on the remaining substrates. A *Rhodococcus erythropolis* pure culture originating from an oil field was able to metabolize n-alkanes with carbon lengths of 10 to 30 as well (Milekhina et al. 1998). Chua et al. (1996) demonstrated *Gordonia amarae* isolated from activated sludge scum to utilize LCFAs with chain lengths up to 24 carbons. In situ studies with PTLO in scum by microautoradiography demonstrated that PTLO were highly selective in their substrate uptake abilities, utilizing only oleic acid among many substrates tested, e.g. monosaccharides, amino acids, different LCFAs, glycerol, and trioleate (Eales et al. 2005). In contrast, pure culture studies of *Skermania piniformis* showed these organisms to be able to utilize glycerol as a carbon source in TYG medium (Blackall et al. 1989). Soddell and Seviour (1996) found out that *Skermania piniformis* produced more biomass and grew faster on hydrophobic substrates like olive oil and Tween 80 than on simple hydrophilic substrates like glucose.

In general an attachment of nocardioform actinomycete cells to hydrophobic compounds rather than growth in the water phase was observed (Soddell et al. 1998). These properties allow competing for nutrients with faster-growing but not hydrophobic organisms in activated sludge. This is corroborated by a study of Lemmer and Baumann (1988a) demonstrating enhanced successful competitional growth of *Gordonia amarae* within the sludge biocenosis in the presence of hydrophobic substrates as compared to controls without hydrophobic substances. Production of biosurfactants and hydrophobic cell wall components reported for nocardioform actinomycetes seem to improve both adhesion and consequently bioavailability of hydrophobic components. The cell wall of nocardioform actinomycetes is rich in covalently bound hydrophobic mycolic acids, which are high molecular bound hydroxy fatty acids with long aliphatic side chains (C30 to C90). These aliphatic chains are not anchored into the cytoplasmatic membrane but exposed to the exterior. Consequently the cell surface is characterized by hydrophobic properties that causes adherence to hydrophobic phases in a two phase system (Neu 1996). This was confirmed by observations of Bendinger et al. (1993) who demonstrated that bacteria with mycolic acids were more hydrophobic than bacteria without. Furthermore a tendency towards an increase in hydrophobicity with increasing mycolic acid chain length is reported. However, in general this effect was smaller than expected and in some cases species with similar carbon length showed various hydrophobic features. Stratton et al. (1998, 2002) demonstrated as well that mycolic acid composition is not the only factor contributing to cell surface hydrophobicity and consequently enhancing scum formation potential of nocardioform actinomycetes. Surface compounds such as glycolipids, exopolysaccharides, proteins, lipoteichoic acids, and peptidolipids might as well increase cell surface hydrophobicity or else decrease hydrophobic effects (Neu 1996).

The production of biosurfactants is well known for nocardioform actinomycetes growing on hydrophobic substrate. These surfactants are predominantly glycolipids (Rosenberg 1986, Ramsay et al. 1988, Soddell and Seviour 1990, Lang and Philp 1998, Pagilla et al. 2002). The species studied most intensely is *Rhodococcus erythropolis*, which produces trehalose-containing glycolipids in the presence of n-alkane (Kretschmer et al. 1982, Kim et al. 1990, Lang and Philp 1998, Milekhina et al. 1998). These trehalose lipids remain cell bound and consist of a mixture of nonionic trehalose-dicorynomycolates, trehalose-monocoryno-mycolates and trehalose-tetraesters (C6 to C12). The production of the various types and quantities of rhodococcal trehalose lipids is depending on the growth conditions (Kim et al.

1990). It was shown that *R. erythropolis* synthesized mostly trehalose-monocorynomycolates and trehalose-dicorynomycolates in the presence of n-alkanes and nitrogen saturation. In contrast, under nitrogen limitation and a temperature shift from 30°C to 22°C an overproduction of trehalose-tetraesters was observed. *Nocardia corynebacterioides* produced a novel nonionic pentasaccharide lipid that represents the major component of the glycolipids (92%) together with the known trehalose-monocorynomycolates and dicorynomycolates during cultivation on n-alkanes (Kim et al. 1990). It is noteworthy that the described glycolipid production for nocardioform actinomycetes is only induced by the presence of hydrophobic carbon sources whereas rhamnolipids as being produced by *Pseudomonas aeruginosa* can also be derived from non-hydrocarbon substrate (Guerra-Santos et al. 1984) and should be regarded as secondary metabolites (Lang and Philp 1998). The role of the biosurfactants produced by nocardioform actinomycetes is generally accepted as to lower the interfacial tension between different phases. This emulsifying effect makes a direct contact between cells and hydrophobic substrate easier that is the prerequisite for substrate to enter the microbial cells (in Bell et al. 1998).

A possible growth strategy of a specific *Rhodococcus* strain isolated from seawater growing on crude oil was investigated by Bredholt et al. (1998). The presence of alkanes induced the formation of a hydrophobic cell surface containing specific polymers with emulsifying activity. This permitted oil-associated exponential growth where an extensive emulsification of the residual oil and accumulation of acidic oxidation products occurred. At the end of the exponential growth phase the most intense emulsifying effect was observed that was caused by a release of cell bound biosurfactants. The cells regained their hydrophilic nature accompanied with an increase of cells in the water phase. During the linear growth phase these free hydrophilic cells consumed the accumulated acidic alkane-oxidation products, which are associated with the oil phase but in equilibrium with water-dissolved compounds. Another mechanism of tolerating and even growing in the presence of oil and n-hexadecane, which might as well have a toxic effect, is the production of extracellular polysaccharides (Iwabuchi et al. 2000). Some *Rhodococcus rhodochrous* strains produced these specific capsular EPS that lower the cell surface hydrophobicity and function as a hydrophilin. These EPS emulsified the hydrocarbons and consequently enhanced the degradation of these components (Iwabuchi et al. 2002). Sunairi et al. (1997) observed that *Rhodococcus rhodochrous* strains that have the capacity to produce EPS were not able to form stable scum layers whereas the strains without EPS characterized by hydrophobic cell surfaces produced scum. The cell wall of both strains had similar mycolic acids components and the differences in cell surface hydrophobicity and scum formation were caused by the ability to produce EPS. A further nocardioform actinomycete with hydrophobic cell surfaces and a strong scum formation potential is *Rhodococcus ruber* (Khan et al. 1991). The excretion of biosurfactants into the medium was reported for this organism during growth on readily degradable carbon sources such as sucrose, yeast, and peptone (Khan and Forster 1988). However, growth rates were enhanced in the presence of straight-chain alkanes and LCFAs (Khan and Forster 1990). This specific polymer consists predominantly of carbohydrate and protein and the surface activity of these components caused the decrease in surface tension.

Lind and Lemmer (1998) found out that nocardioform actinomycetes were frequently present in WWTPs with moderate and high F/M ratio (0.1 to 0.7 kg BOD_5 kg^{-1} MLSS d^{-1}). Growth was favored in the presence of high amounts of lipids and surfactants in the wastewater (in

Lemmer and Lind 2000). Especially particulate substrates - non-hydrolyzed substances - promote the development of high numbers of nocardioform actinomycetes. Various data about actinomycete-promoting sludge age from 1 to 30 d are published (in Lemmer and Lind 2000). In most of these studies it was not differentiated between mixed liquor and scum layer retention time leading to contradictory information about both sludge age and sludge load. Furthermore the following parameters might promote optimal growth of nocardioform actinomycetes: industrial wastewater components (poorly degradable hydrocarbons), high oxygen concentration in the aeration tank and a high sludge circulation (Lind and Lemmer 1998). In WWTPs operated with pre-denitrification nocardioform actinomycetes were more frequently found as compared to alternating or intermittent nitrogen removal systems. Simultaneous phosphorus precipitation has a positive effect on the development of nocardioform actinomycetes in comparison to pre- and post-precipitation.

2.3.2.3 Other filamentous bacteria in scum

Nostocoida limicola was first isolated from activated sludge, described, and named by van Veen (1973). The organism is a slow growing Gram-positive bacterium forming filaments with a length of 600 to 900 µm consisting of spherical to discoid cells. Variations of the morphological features such as cell diameter, cell length, filament length, and visibility of septa between the individual cells make a sound identification of *N. limicola* questionable. Eikelboom and van Buijsen (1983) proposed three morphotypes of *N. limicola*, with *N. limicola* I exhibiting the smallest cells and filament lengths and *N. limicola* III the largest cells and filaments. They assumed *N. limicola* to be a single bacterium existing in activated sludge in several morphological variations.

Recent 16S rDNA sequence analyses of several *N. limicola* isolates have shown that *N. limicola* belonged at least to five different phylogenetic lineages. The morphotype ***N. limicola* I** isolated from Czech and Australian WWTPs consists of at least two genera, *Trichococcus* and *Streptococcus* within the *Firmicutes* phylum (Liu et al. 2000). A high diversity was determined for ***N. limicola* II**. Five different Australian isolates were phylogenetically located within the *Actinobacteria* phylum closely related to *Terrabacter* spp.. However, they probably belong to a novel genus (Blackall et al. 2000). Because of the lack of sufficient chemotaxonomic data and of essential information about DNA composition the organism is referred to as "*Candidatus* Nostocoida limicola". Further isolates from a German municipal WWTP were classified within the *Chloroflexi* phylum (formerly green non sulfur bacteria) with *Sphaerobacter thermophilus* as the nearest published relative, however, with a low sequence similarity of 87% (Schade et al. 2002). Isolation and cloning techniques were carried out to obtain phylogenetic information about *N. limicola* II morphotype originating from different industrial WWTPs (textile industry, brewery and paper mill, Snaidr et al. 2002). The phylogenetical 16S rDNA analyses of the isolates and clones revealed an affiliation to the *Alphaproteobacteria* only distantly related to any previously described bacteria (<93% similarity to *Rhodospirillum* spp.). For these novel species the following names "*Candidatus* Alysiosphaera europaea", "*Candidatus* Alysiomicrobium bavaricum", and "*Candidatus* Monilibacter batavus" were proposed (Levantesi et al. 2004). ***N. limicola* III** isolates were classified within the *Planctomycetes* phylum, most closely related to *Isosphaera pallida* (Liu et al. 2001). However, phenotypic differences were observed, e.g. *N. limicola* III isolates did not glide and were not thermotolerant. For all phylogenetically characterized *N.*

limicola described specific rRNA-targeted oligonucleotide probes were designed (Liu and Seviour 2001, Schade et al. 2002, Snaidr et al. 2002). A FISH survey of different WWTPs all over the world (Australian and various European WWTPs) was carried out by Liu and Seviour (2001), where *N. limicola* morphotypes were detected in activated sludge with their own specific probes for *N. limicola*. The results demonstrated a high diversity of the morphotype *N. limicola*, and the most frequently detected morphotype was *N. limicola* II that belonged to the *Actinobacteria* ("*Candidatus* N. limicola") and to the *Alphaproteobacteria*.

In accordance with the taxonomic heterogeneity of the morphotype *N. limicola* different physiological properties were observed. The first *N. limicola* isolates were aerobic, facultatively anaerobic or microaerophilic organisms and grew at 11 to 35°C with an optimum growth at 30°C (van Veen 1973). Later studies by Nowak and Brown (1990) found similar results concerning oxygen demand and growth temperature. The *N. limicola* filaments were Gram- and Neisser-positive, and polyhydroxybutyrate (PHB) granules were frequently observed inside the cells. Furthermore these *N. limicola* isolates were able to use a variety of substrates such as glucose, saccharose, glycerol, and Tween 80 if peptone was present in the media as carbon and energy sources. Poor growth was observed on acetate and citrate even if peptone was supplemented. In contrast, Blackall and co-workers (2000) isolated Gram-variable *N. limicola* II strains identified as "*Candidatus* N. limicola" that showed optimal growth on both R2A medium and synthetic medium containing different carbon sources such as glucose, acetate, glycerol, and Tween 80. None of these strains could grow without added nitrogen, reduce nitrate to nitrogen gas or grow anaerobically, whereas all could grow at 15 to 30°C and reduce nitrate to nitrite. Some cells produced lipophilic inclusions. In situ studies by microautoradiography demonstrated under aerobic conditions that at two different WWTPs *N. limicola* morphotypes exhibited high uptake rates of glucose and oleic acids, and only at one WWTP *N. limicola* showed a minor uptake of acetate, glycine, and leucine (Andreasen and Nielsen 1997). The anaerobic uptake of oleic acid supports the hypothesis *N. limicola* to be active under anaerobic conditions. These findings indicate that the in situ detected *N. limicola* might be the same organisms that were isolated by van Veen (1973) and later by Nowak and Brown (1990). They differ from the Blackall et al. (2000) isolates. Schade and Lemmer (2002) isolated three Gram-positive *N. limicola*-like strains that showed 98.5% 16S rDNA sequence similarity to "*Candidatus* N. limicola" and showed similar physiological properties. The utilization of different carbohydrates such as mono-, di-, and polysaccharides under aerobic conditions was optimized in the presence of ammonium. A distinctly lower growth was observed with nitrate. Therefore it is not surprising that this organism was often found in WWTPs characterized by wastewater containing high amounts of saccharides without full nitrification (Schade and Lemmer 2002). Further Gram-variable *N. limicola*-like strains classified within the *Chloroflexi* phylum were isolated by Schade et al. (2002) and showed a totally different growth behavior. These strains grew well in sludge hydrolysate medium at COD values between 1 to 4 g L^{-1} and at a pH range from 6 to 7.8 under aerobic conditions. No growth was observed in R2A medium and in several synthetic mineral media containing various carbon sources (glucose, acetate, Tween 80, and citrate) and nitrogen sources (NH_4^+, NO_3^-, and peptone). These findings indicate that the *Chloroflexi*-related *N. limicola* needs a complex nutrient supply for optimal growth in contrast to the *Actinobacteria*-related *N. limicola*, which was able to grow well on simpler substrates. Little is known about the cell surface properties of *N. limicola.* However, Goddard and Forster (1987) demonstrated that

this organism is able to produce biosurfactants in the presence of hydrophobic substrate. These biosurfactants consisted of lipids, lipopeptides, proteins, and carbohydrates, and had a high emulsifying and foaming potential.

N. limicola prefers a moderate sludge load with an F/M ratio of 0.2 to 0.3 kg BOD_5 kg^{-1} MLSS d^{-1} (Lind and Lemmer 1998). According to Seviour et al. (1994) *N. limicola* in general was detected in low loaded WWTPs with a temperature above 15°C and a sludge age above 20 days. Similar observations were found by Scruggs and Randall (1998) who described *N. limicola* organisms to grow at a very low F/M ratio (0.02 to 0.1 kg $COD_{dissolved}$ kg^{-1} SS d^{-1}). These contradictory findings indicate that different organisms constitute morphotype *N. limicola* as was discussed above. *N. limicola* was in general found in nutrient removal plants together with *M. parvicella* (Andreasen and Nielsen 1997). However, in contrast to *M. parvicella,* a selective growth of *N. limicola* was observed in WWTPs operated with pre-denitrification as compared to alternating or intermittent nitrogen removal (Lind and Lemmer 1998). Furthermore *N. limicola* favors wastewater without mechanical pre-treatment. Scruggs and Randall (1998) showed that *N. limicola* growth was not dependent on a high oxygen concentration but was stimulated by occasional anaerobic or very low dissolved oxygen concentrations. These findings confirm the pure culture and in situ studies described above.

The filamentous bacteria types 0092, 0041/0675, and 1851 were first described and named by Eikelboom (1975). **Type 0092** develops filaments with 0.4 to 0.6 µm in diameter and the total filament length is below 200 µm. Neisser staining turns the organism gray-black throughout the filament. The filaments of type 0092 in general extrude from the flocs or are entangled within the floc particles but are rarely found free-floating in the liquid phase between the flocs. The first 0092 isolate is reported to be an aerobic organism that hydrolyzes gelatin, casein, and urea but not starch (Horan et al. 1988). In a subsequent study (Buali and Horan 1989) the growth kinetics of type 0092 and its behavior under feed and starvation conditions was examined. During the starvation phase a disintegration of the filamentous morphotype into individual cells was observed, which was described to be a reversible process. Further 0092 morphotypes were isolated by Bradford et al. (1996) that showed optimal growth on R2A agar. This organism was phylogenetically analyzed by 16S rDNA sequence analysis and fell within the *Flavobacterium* subgroup (formerly *Cytophaga*) closely related to *Flavobacterium columnare* (formerly *Cytophaga columnaris*, 96.6% sequence similarity). Schade and Lemmer (2002) cultivated type 0092 isolates on R2A and sludge hydrolysate medium. However, these organisms survived in pure culture for a short period only so that they could not yet be physiologically characterized. It was suggested that these 0092 isolates were different from the *Flavobacterium*-related type 0092 isolates because of their variation in colony morphology. This was confirmed by negative FISH signals with a specific probe for a *Flavobacterium*-related 0092 isolate designed by Bradford (1997). Moreover these organisms were not even detectable by probes specific for the domain *Bacteria* (EUB338 probes, Amann et al. 1990, Daims et al. 1999) pointing out that this organism might belong to a new not yet described lineage within the domain *Bacteria*. In the literature different F/M ratios (0.02 to 0.26 kg BOD_5 MLSS kg^{-1} d^{-1}) are discussed to promote growth of type 0092 in WWTPs (in Lemmer and Lind 2000) indicating various organisms to constitute morphotype 0092. Temperature seems to have a small effect on the development of type 0092 (Seviour et al. 1994). Low oxygen concentration favors the occurrence of type 0092 as was shown in a screening by Lind and Lemmer (1998). The same study demonstrated

that type 0092 was in general found in low loaded nutrient removal plants with aerobic, anaerobic, and anoxic zones. Furthermore type 0092 prefers raw wastewater with a high percentage of industrial wastewater. Eikelboom et al. (1998) showed type 0092 to compete for the particulate COD wastewater fraction together with *M. parvicella*, nocardioform actinomycetes, type 0041, and type 1851. During the summer season type 0092 often replaces *M. parvicella*. In contrast to *M. parvicella* type, type 0092 is not dependent on reduced nitrogen components and therefore might find competitive growing conditions during complete nitrification in summer (Eikelboom et al. 1998).

Type 0041 is characterized as straight or slightly bent Gram-positive filamentous organism with a filament length up to several hundred µm that is surrounded by a sheath (Eikelboom 1975). **Type 0675** shows a close resemblance to type 0041 but exhibits a smaller cell size. Nevertheless it is difficult to differentiate between both morphotypes and for that reason at present they are summarized as **type 0041/0675**. The attached growth makes it difficult to isolate these organisms. Therefore no 16S rDNA data are available and only little is known about their physiological properties. However, application of FISH with specific probes for candidate division TM7 of the domain *Bacteria* detected the morphotype 0041/0675 (Hugenholtz et al. 2001). Thomsen et al. (2002) demonstrated that approximately 15% of 0041 filaments belonged to the TM7 group whereas all other 0041 filaments hybridized with the general bacterial EUB338 probes only. None of the other probes applied for the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria,* and *Bacteroidetes* gave positive signals. These results indicate the phylogenetic heterogeneity of the morphotype 0041. In the same study in situ substrate uptake experiments by microautoradiography were carried out. No significant physiological differences were found between the TM7-positive and TM7-negative 0041 filaments. It was shown that most type 0041 took up glucose under aerobic, anoxic, and anaerobic conditions, but none of the 0041 filaments was able to consume acetate under any condition. Many 0041 filaments were able to take up the amino acid leucine, while only a few could take up the amino acid glycine under aerobic conditions. For type 0041 no uptake of oleic acid was observed by Thomsen et al. (2002), whereas Andreasen and Nielsen (1997) detected a weak uptake rate. Type 0041/0675 is able to store polyhydroxybutyric acid but no polyphosphate deposit was observed (Williams and Unz 1985). According to Goddard and Forster (1987) type 0041 is able to produce biosurfactants, which might favor scum formation. Different studies showed that type 0041/0675 occurred at a wide range of F/M ratios from 0.03 to 0.3 kg BOD_5 kg^{-1} MLSS d^{-1} (in Lemmer and Lind 2000). Type 0041/0675 needs a high oxygen content, however, changes of aerated and non-aerated zones support the growth of these organisms (Lind and Lemmer 1998). Nitrogen and phosphorus deficiency promote the development of type 0041/0675 as well (Jenkins et al. 1993). This explains the frequent occurrence of these organisms in nutrient removal plants (Lind and Lemmer 1998). Type 0041/0675 was found more often to dominate the microbial biocenosis in nutrient removal plants with pre-denitrification and simultaneous phosphorus precipitation as compared to plants with intermittent/alternating nitrogen elimination and pre-/post-phosphorus precipitation. Furthermore type 0041/0675 prefers non-treated raw wastewater where hydrolysis processes have not yet taken place.

Type 1851 is another sheathed filamentous organism with attached growth (Eikelboom 1975). The organism is Gram-positive but the filaments do not stain intensely blue. The filaments with 0.5 to 0.7 µm diameter and 200 to 300 µm length are straight or slightly bent and rise

from the sludge flocs often developing bundles. Because of the attached growth it is difficult to obtain pure cultures of type 1851. Nevertheless two different work groups (Beer et al. 2002, Kohno et al. 2002) were recently able to isolate type 1851 from activated sludge, and physiological and phylogenetic investigations were carried out. The isolates of both studies were grouped within the *Chloroflexi* phylum according to their 16S rDNA sequences. Its closest relative is *Roseiflexus castenholzii* but with 84% sequence similarity only. This indicates that the type 1851 isolates might have to be described as a new genus within the *Chloroflexi* phylum. Björnsson et al. (2002) demonstrated by in situ studies using FISH the occurrence of filamentous *Chloroflexi* belonging to subdivision 3, which includes type 1851 isolates described above, to be abundant in activated sludge. Beer et al. (2002) designed the probe CHL 1851 specific for these type 1851 isolates. The application of FISH on activated sludge with type 1851 filaments from several WWTPs (Australia, New Zealand, the Netherlands and France) demonstrated that type 1851 responded positively to probe CHL 1851. It was suggested that type 1851 is phylogenetically very similar regardless of its location. Physiological analyses showed positive polyhydroxybutyric acid staining and in general negative Neisser staining, which indicates that no polyphosphate is stored by the type 1851 isolates tested (Kohno et al. 2002). The isolates were able to utilize pyruvate and various sugars including glucose, fructose, mannose, maltose, trehalose, melibiose, raffinose, arabinose, and xylose as carbon and energy sources. The development of type 1851 in WWTPs is favored by similar operating conditions as was discussed for the other filamentous scum bacteria such as low sludge load, changes of aerobic, anoxic, and anaerobic zones within the same reactor and a high amount of particulate COD in the influent (in Lemmer and Lind 2000). Pre-denitrification favors growth of type 1851 as compared to intermittent/alternating nitrogen elimination.

2.3.2.4 Non-filamentous bacteria

In most reports scum is associated with the occurrence of high numbers of filamentous bacteria. Nevertheless some studies report non-filamentous bacteria to represent the main constituent in scum. Seviour et al. (1990) detected unidentified Gram-positive coccoid cells that could be the coccoid stage of *Rhodococcus* spp.. However, it is still little known about this metamorphosis.

Non-filamentous hydrophobic bacteria were isolated from scum, identified, and characterized with respect to nutrient requirement, hydrophobicity, and emulsification ability (Lemmer et al. 1998). The isolates belonged to 4 main groups, nocardioform actinomycetes, *Beta*- and *Gammaproteobacteria*, and *Cytophaga/Flavobacterium*. *Rhodococcus* spp. were able to use different carbon (acetate, ethanol, and Tween 20), nitrogen, and phosphorus sources. Their hydrophobicity is in general high for most growth conditions investigated. Excretion of biosurfactants was observed during the post-exponential growth phase. Further isolates identified as *Hydrogenophaga pseudoflava* and *Acidovorax facilis* showed in general weak growth on the carbon substrates tested. Emulsifiers were produced during growth on R2A medium. Cell surface hydrophobicity was strain-dependent for *Hydrogenophaga* spp. and low for *Acidovorax* spp.. The third group was *Acinetobacter* spp. with *A. calcoaceticus* as the dominant organism. These organisms showed optimal growth on acetate and some strains of *A. calcoaceticus* used Tween 20 as a carbon source. High hydrophobicity was determined during growth on R2A whereas moderate hydrophobicity was detected on acetate and ethanol.

Biosurfactants were produced only with ethanol and seemed to be substrate-dependent. The ability of *Acinetobacter* spp. to degrade hydrocarbon, to develop a high cell surface hydrophobicity and to excrete surface-active material is well documented in the literature (Rosenberg 1986, Towner 1992, Zita and Hermansson 1997b, Lang 1999). These properties of *Acinetobacter* might contribute to form stable scum layers. Isolates from the fourth group, the *Cytophaga/Flavobacterium* group, were characterized by low cell surface hydrophobicity and by emulsification capacity with several substrates. In situ studies (Müller et al. 2002) applying FISH with genus- and group-specific probes for the described species demonstrated that the impact of these non-filamentous organisms on scum formation might have been overestimated except for non-filamentous nocardioform actinomycetes. This was confirmed by other work groups, who showed short irregular rods identified as nocardioform actinomycetes to be abundant in activated sludge or scum (Schuppler et al. 1998, Davenport et al. 2000, Hug et al. 2005). No in situ identification on genus and species level is reported for these non-filamentous nocardioform actinomycetes. Therefore it is not known whether these organisms represent a growth variation of the branched filamentous organisms or else different species.

2.4 Microbial Community Analyses in Activated Sludge

The microbial activated sludge communities have long been regarded as “black box”. Today, even engineers agree a precise knowledge of the structure and function of this complex bacterial biocenosis to be a prerequisite for further plant optimization. The standard methods for monitoring microbial biocenoses in WWTPs have been microscopic analysis and cultivation techniques, and only in the last years novel molecular methods have been applied to characterize the community structure in wastewater systems.

The microscopic sludge analysis according to Eikelboom (1975, 2000) represents the classical approach for the identification of filamentous bacteria by transmission and phase contrast microscopy. This method failed to characterize filamentous bacteria with morphological and taxonomical variations and non-conspicuous microorganisms such as nitrifiers or polyphosphate accumulating organisms (PAO). Further identification tools such as isolation and cultivation techniques followed by physiological/biochemical characterization as well as molecular biological approaches are consequently required. It was shown that cell numbers determined by cultivation-dependent methods as colony forming units (CFU) revealed in general distinctly lower values in comparison to microscopic total direct cell counts. Only 0.1% of the total bacteria in seawater (Kogure et al. 1980, Ferguson et al. 1984), only 0.25% of the total bacteria in freshwater (Jones 1977) and not more than 15% of the total bacteria in activated sludge (Wagner et al. 1993, Kämpfer et al. 1996) were found to be cultivable. These observations indicate that the remaining bacteria are not able to grow at the substrate and milieu conditions used for isolation and cultivation. The studies performed on activated sludge demonstrated that nutrient-rich media favored the growth of heterotrophic saprophytes and selected against other bacteria present in much higher numbers in activated sludge. The significant differences between total cell counts and the fraction of cultivable bacteria in environmental samples are today well known as the “great plate count anomaly” (Staley and Konopka 1985). The reasons for this phenomenon are (i) little knowledge about growth requirements of environmental microorganisms and (ii) the dormant “viable but non

culturable" (VBNC) stage, where bacterial cells are intact and alive but do not undergo cell division (Roszak and Colwell 1987). VBNC stage of bacterial cells has been observed for many aquatic and marine habitats, and reflects a survival strategy of bacterial cells at poor environmental conditions, e.g. nutrient deficiency and low temperature. These findings point out that culture-based techniques are inadequate for studying bacterial diversity from environmental samples. Molecular approaches for the cultivation-independent detection of microorganisms have been developed and a suitable combination of these techniques allows to analyze the composition of natural microbial populations (see review by Amann et al. 1995).

2.4.1 Classical Microscopic Investigations

The microscopic activated sludge analysis was developed by Eikelboom in the 1970th (Eikelboom 1975) and refined during the last years (Eikelboom 2000). This analysis comprises the description of sludge flocs including floc morphology, composition of the flocs (microbial diversity, (in)organic particles), and the detection of free cells, e.g. spirochetes and *Spirillum* spp.. Furthermore protozoa, e.g. flagellates and ciliates, as well as metazoa, e.g. nematodes, are determined. Moreover conspicuous bacteria in particular filamentous bacteria are described. The results of an activated sludge analysis give important information about the sludge quality and its environmental conditions, which in turn is used to monitor and influence operating conditions.

The identification of filamentous bacteria is based on determination of morphology of the individual cell and the whole filament, gliding motility, and staining properties such as Gram- and Neisser-staining. Twenty-six filament types were distinguished and grouped into seven assemblages with about 10-15 of these types being frequently found in activated sludge. As most of these microorganisms are not yet taxonomically classified properly, they were named by numbers, e.g. type 0041, type 1863, or even taxonomically invalid names, e.g. *Microthrix parvicella* and *Nostocoida limicola*.

The population size of filamentous organisms can vary strongly. Therefore process monitoring includes quantification of these organisms. It is possible to determine the precise number and length of the filaments by counting and using specific measuring methods. However, this approach is very time-consuming and is only possible for straight filaments. Strongly curled filaments numbers are not recordable. Therefore for routine analysis the filament index (FI) was proposed to apply. The FI describes the filamentous population size using categories from 0 (no filaments) to 5 (extensive growth of filaments) with the individual categories differing by a factor of 10. An FI of 1 or 2 indicates filamentous organisms to have only a slight effect on sludge settleability. A deterioration of sludge settleability is observed at FI 3 and higher with concomitant development of bulking sludge. However, it has to be kept in mind that in addition to the number of filamentous organisms, other parameters such as filament type and the activated sludge EPS (see chapter 2.2.2) might affect sludge settleability. The filamentous bacteria populations in general consist of various bacteria. However, usually these filament types do not occur in a similar amount. Often one filamentous bacterium dominates the biocenosis ("dominant" filamentous organism) whereas other filamentous bacteria are found in distinctly lower numbers ("secondary" filamentous

organisms). Organisms are called “secondary” filamentous bacteria when they represent 5 to 10% of the amount of the “dominant” filamentous organism.

The described classification system of filamentous bacteria has been widely adopted and made it possible to carry out comparative studies in different WWTPs all over the world. Experience from classical microscopic sludge analysis in the last years provides information about wastewater composition, process stability, operating conditions, e.g. oxygen concentration and sludge load, and specific sludge problems, e.g. bulking and scum formation. Based on these results the wastewater treatment process was optimized by specific technical countermeasures.

2.4.2 Molecular Biological Techniques

Molecular biological approaches based on molecular markers such as ribosomal RNA (rRNA) or its encoding gene, the ribosomal DNA (rDNA), proved to strongly support identification and enumeration of the microbial community within activated sludge. The most frequently used phylogenetic markers are the 16S (prokaryotes) and 18S (eukaryotes) small subunit rRNAs.

Bacterial 16S rRNA genes are retrieved from environmental samples by DNA extraction and amplification using polymerase chain reaction (PCR) with specific 16S rDNA primers, which provides enough rDNA templates for further investigations. A separation step such as cloning technique (clone libraries, see chapter 2.4.2.2) or denaturing gradient gel electrophoresis (DGGE, see chapter 2.4.2.3) is required for the amplified environmental DNA because various DNA fragments originating from different organisms characterize it. Sequencing of DNA fragments followed by comparative sequence analysis allows phylogenetic classification. However, only the application of fluorescent in situ hybridization (FISH) with specific rRNA-targeted oligonucleotide probes verifies the occurrence of these organisms in the original sample (see chapter 2.4.2.4).

Furthermore the isolation of conspicuous organisms by micromanipulation followed by DNA extraction, amplification of rDNA, and sequence analysis without cultivation provides phylogenetic information about filamentous organisms present in activated sludge. On the basis of these data it is possible to design oligonucleotide probes and identify these organisms by FISH in situ in the activated sludge floc (Blackall et al. 1994b, Bradford 1997, Erhart et al. 1997, Liu and Seviour 2001, Beer et al. 2002, Schade et al. 2002).

2.4.2.1 Comparative sequence analysis of ribosomal RNA (rRNA)

Comparative sequence analysis of rRNA sequences is one of the most important methods for the elucidation of bacterial phylogeny as well as bacterial identification (Woese 1987, Ludwig and Schleifer 1994, Ludwig et al. 1998). Over the last years extensive databases have been created. For the 16S rRNA gene (16S rDNA) more than 150,000 bacterial 16S rDNA sequences with cumulative data are available from the core databases that are accessible to all researchers (EMBL-Bank, United Kingdom, Kulikova et al. 2004, DNA Databank of Japan, DDBJ, Miyazaki et al. 2004, GenBank, USA, Benson et al. 2005). The availability of huge rDNA databases nowadays provides optimal conditions for phylogenetic analysis, which

allows not only the definition of phylogenetically related organisms but also recognition of molecular signatures for these groups (Woese 1987).

The rRNA molecules are useful molecular markers for studying phylogenetic relationships because they are present in all bacteria, are functionally constant, and are composed of highly conserved as well as more variable domains (Woese 1987, Ludwig and Schleifer 1994). Comparative analyses of rRNA sequences identify so-called signature nucleotides or nucleotide motifs on various taxonomic levels that are perfect targets for an evolutionary identification. Signatures are primary structure elements, e.g. single nucleotides or sequence stretches, insertions (Roller et al. 1992), and deletions (Ludwig and Schleifer 1994). Furthermore the presence and shape of higher order structure elements (helices) often correlate with higher and lower phylogenetic entities (Ludwig et al. 1992, Roller et al. 1992). The analysis of these marker molecules demonstrated that rRNA sequences provide sufficient data to determine relationships between genera, families, and other higher ranked taxa but also for species delineation (Vandamme et al. 1996). However, Fox et al. (1992) reported for *Bacillus* strains that 16S rRNA sequence identity might not be sufficient to guarantee species identity. Despite being sufficient for reliable phylogenetic analysis rRNA marker molecules provide only limited information and consequently a limited resolution power, which allows only a spot check of the evolutionary history of microorganisms (Ludwig and Schleifer 1994, Ludwig et al. 1998).

Primary structure signatures can be used as target sites for diagnostic hybridization probes and consequently identify microorganisms on the basis of their phylogenetic relationship (Amann and Ludwig 1994). Probes can be designed for groups of different phylogenetic depths (Ludwig and Schleifer 1994) and were successfully applied for domains (Amann et al. 1990), phyla (Roller et al. 1994), classes (Manz et al. 1992), genera and species (de los Reyes et al. 1997). However, the sequence variation of rRNA molecules is usually not sufficient to design strain-specific probes.

2.4.2.2 Full-cycle rRNA approach

The full-cycle rRNA approach includes most of the steps described above such as DNA extraction, amplification, standard molecular techniques to obtain a clone library and to retrieve rDNA sequence information, and performance of comparative sequence analysis. Finally sequence-specific oligonucleotide probes are to be designed and evaluated by FISH to identify and enumerate in situ the bacterial biocenoses in the original samples (see review by Amann et al. 1995).

After amplification the cloning technique separates each rDNA fragment from the others by ligation of the PCR product into vector molecules, which are afterwards transformed into a host cell of a specific type of *Escherichia coli*. In general each transformed *E. coli* cell contains only one modified plasmid molecule and is now known as a “clone”, and can be separately stored for further analysis. The collection of high numbers of clones (clone library) provides a high amount of different rDNA sequences. Further analysis involves determination of either full or partial 16S rDNA sequences from bacteria stored in the plasmid of the host cells. The performance of a comparative sequence analysis of these sequences yields information on the identity or relatedness of new sequences in comparison with available

databases and gives an estimation of the genetic diversity in the samples examined. The described technique resulted in the detection of high numbers of mostly uncultivated bacteria in various habitats such as activated sludge (Bond et al. 1995, Schuppler et al. 1995, Snaidr et al. 1997), soil (Borneman and Triplett 1997), and contaminated aquifers (Dojka et al. 1998). Furthermore this approach discovers previously unknown bacterial taxa up to the level of new phyla (Liesack and Stackebrandt 1992, Hugenholtz et al. 1998, Hugenholtz et al. 2001).

However, it has to be kept in mind that sequence analysis of the clone library gives no information about the identified organisms to be really present in the original sample and about their abundance. Furthermore organisms that occur quite frequently within the microbial biocenosis of the sample examined might not be detected by sequence analysis. A possible reason for missing sequences of organisms that are present in original samples is that the used DNA extraction protocol is not effective in breaking open all bacterial cells (Kuske et al. 1998). In addition, bacteria that are not targeted by the PCR primers applied will not be detected by rRNA gene sequence analysis. Furthermore sequences might relate to organisms that are not found in the original samples because of contamination of the samples or reagents used. This deposit of DNA from allochthonous organisms can function as PCR template (Tanner et al. 1998). Finally, it was shown by different research groups (Suzuki and Giovannoni 1996, Polz and Cavanaugh 1998, Suzuki et al. 1998) that the relative abundance of amplified rDNA genes in the clone library does not necessarily represent the gene ratios of the original DNA. Therefore rRNA sequence analysis has to be completed by other techniques, e.g. FISH combined with microscopy and digital image analysis, that visualize and quantify the microbial cells in situ. In activated sludge the full-cycle rRNA approach was applied, e.g. for characterization of the polyphosphate-accumulating organisms (PAO) in EBPR (Crocetti et al. 2000) and nocardioform actinomycetes in activated sludge scum formation (Schuppler et al. 1995, Schuppler et al. 1998).

Up till now, the full-cycle rRNA approach is the most successful method to investigate microbial diversity and to determine the species composition of mixed microbial biocenoses. However, in general these studies have focused only on the exploration of microbial diversity and have not given any information on the complex dynamics, which microbial communities develop by seasonal fluctuation or after environmental changes such as contamination with toxicants. For this purpose the cloning approach is not useful, because it is quite time-consuming and laborious, and therefore impractical for multiple sample analysis. Population shifts might be analyzed, e.g. using FISH with taxon-specific probes. This method, however, is only able to analyze microorganisms for that specific probes are available. Therefore other molecular biological techniques such as nucleic acid fingerprinting might be better suitable to study the complex structure of microbial communities and their dynamics.

2.4.2.3 Nucleic acid fingerprinting

Nucleic acid fingerprinting (genetic fingerprinting) methods provide a pattern or a profile of the community diversity based upon physical separation of unique nucleic acid species. The methods are rapid and relatively easy to perform and consequently the analysis of high numbers of samples is possible. This is necessary for the comparison of the genetic diversity of microbial communities form different environments, or to study the behavior of individual communities over time. In the following only two nucleic acid fingerprinting methods, the

restriction fragment length polymorphism (RFLP) and the denaturing gradient gel electrophoresis (DGGE), are described. For both methods first the molecular marker, i.e. the 16 rDNA, has to be gained by DNA extraction from the environmental samples and by amplification using the PCR technique with 16S rDNA primers.

RFLP of PCR-amplified rDNA, the amplified rDNA restriction analysis (ARDRA), generates nucleic acid profiles after the digestion of the rDNA with restriction enzymes. The restriction endonucleases cut the PCR amplicon at defined sequences and produce linear fragments developing specific patterns after gel electrophoresis and ethidium bromide staining. ARDRA was used to monitor the prokaryotic diversity in hypersaline ponds (Martinez-Murcia et al. 1995) and microbial community shifts after copper contamination (Smit et al. 1997). However, the estimation of microbial diversity by ARDRA is problematic because the number of bands in the profile is larger than the number of amplified DNA fragments and an overestimation of the community diversity is possible. It is known that the sequence of one organism in general generates more than one band and consequently the number of bands is not directly related to the number of community members. The ARDRA method is more suitable to characterize pure cultures (Brim et al. 1999) or to organize clone libraries into different taxonomic groups (Burrell et al. 1998).

In contrast to conventional electrophoresis that allows the separation of DNA fragments based on their size, DGGE (Muyzer et al. 1993, Muyzer and Smalla 1998) enables the separation of DNA fragments of identical length but different sequence. Separation in DGGE is based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamid gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide). The sequence variant of double-stranded DNA fragments is characterized by a specific melting temperature (so-called melting domains) that is defined as the temperature where half of the double-stranded DNA molecule is dissociated (“melted”) into single-stranded molecules. The melting temperature depends on different factors such as the percentage of the GC content of the sequence, the length of the sequence, and concentration of DNA denaturants. In contrast to the temperature gradient gel electrophoresis approach (TGGE, Muyzer and Smalla 1998), where a gradient of temperature influences the dissociation of the DNA, the DGGE technique affects the melting of the DNA molecules by denaturants. Once a sequence reaches its melting temperature at a particular position in the DGGE gel, a dissociation of a double helical to a partially single-stranded DNA molecule occurs (partial denaturation), and migration of the molecule stops. DNA fragments characterized by sequence variation consequently stop migrating at different positions in the denaturing gradient whereby they can be effectively separated by DGGE.

During the last years the DGGE technique has become a routinely applied method in many laboratories. This technique is used, e.g. to describe the genetic diversity of *Actinobacteria* in soil (Heuer et al. 1997), to study population dynamics in microbial communities (Ferris and Ward 1997, Santegoeds et al. 1998), and to screen clone libraries for redundancy (Kowalchuk et al. 1997, Hovanec et al. 1998). Bands of interest can be eluted from the gel and sequenced to identify the corresponding bacterial species. Furthermore the application of FISH with specific probes (published probes or newly designed probes based on the sequence information) might verify and quantify the presence of particular microbial populations. One of the disadvantages of the DGGE technique is the separation of relatively short DNA

fragments (approximately 500 bp) and consequently the limitation of available sequence data for phylogenetic analysis and probe design. Furthermore the separation of fragments with different sequences is sometimes not possible, because of similar melting behavior of the fragments. In addition, the determination of community diversity might be difficult in the presence of double bands, which are caused by primer degeneracies or of heteroduplex molecules.

2.4.2.4 Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes

Oligonucleotides are short strands of nucleic acids usually 15 to 30 nucleotides in length that are complementary to 16S rRNA or 23S rRNA sequence regions with highly conserved or more variable positions and consequently characteristic for phylogenetic entities, e.g. domains, phyla, families, genera, and species. These oligonucleotide probes are able to hybridize (to form double-stranded structures) to the specific 16S rRNA or 23S rRNA targets under specific conditions and have been successfully used for the identification of bacteria (Manz et al. 1992). High numbers of target molecules are necessary in the analyzed samples, because no amplification step for the nucleic acid molecules is used before detection. Therefore this method is limited to rRNA analysis because only rRNA is present in sufficient amount in microbial cells.

Two types of rRNA-based hybridization techniques are frequently used in environmental microbiology. Quantitative dot blot hybridization (Stahl et al. 1988, Raskin et al. 1994) is based on complete cell lysis, rRNA isolation, and quantification of distinct rRNA molecules bound to a specific membrane as a “dot” by hybridization with radioactively labeled probes. These data of relative rRNA abundance cannot be translated into cell numbers. In contrast, during fluorescence in situ hybridization (FISH, Amann et al. 1995) the target rRNA molecules remain in the whole fixed cell, the probes diffuse into the cells, bind specifically, and the probe-labeled fluorochromes can be visualized and correlated to single cells by epifluorescence microscopy. Quantification can be performed by manually counting single cells. Moreover relative amounts can be automatically calculated by relating area fractions of specific organisms to the one of “the total of bacteria” defined as cells belonging to the domain *Bacteria* (Daims et al. 2001b). A semi-automatic method quantifying absolute bacterial cell numbers was developed by Daims and co-workers (2001b), and is based on area fractions determination using a known amount of *E. coli* cells as an internal standard. Confocal laser scanning microscopy (CLSM) provides sufficient optical resolution especially along z-axis as to allow optical sections through an object. In combination with digital image analysis it is a successful tool to reveal (i) quantitative data and (ii) information about the microbial structure in a 3 dimensional environment, e.g. an in situ image of a non-destroyed activated sludge floc.

The FISH technique has become a successful tool in microbial ecology over the last decade and the numbers of rRNA-targeted oligonucleotide probes published has been continuously extended (details on published oligonucleotide probes are available at probeBase, Loy et al. 2003). An increasing resolution of the bacterial community structure can be obtained by differentiation of the various bacterial populations by the application of probes with nested specificity (top to bottom approach, Amann et al. 1995). This approach starts with applying

existing probes for the highest taxonomic level, e.g. the domain *Bacteria*, continues with more specific probes for intermediate levels, e.g. *Actinobacteria*, or else *Alpha-*, *Beta*, and *Gammaproteobacteria*, and is concluded using genus- or species-specific probes. For characterization of the lower taxa often a full-cycle rRNA approach might be useful to reveal information about new or so far non-detected bacteria. In the literature numerous studies on the microbial biocenoses in WWTPs analyzed by the FISH technique are available. FISH was used to examine (i) the high bacterial diversity in activated sludge (Manz et al. 1994, Kämpfer et al. 1996, Snaidr et al. 1997) and (ii) to monitor defined groups of bacteria involved in specific processes such as nitrification (Juretschko et al. 1998, Daims et al. 2001a), EBPR (Crocetti et al. 2000, 2002), and denitrification (Neef et al. 1996, Ginige et al. 2004). In the last years a great amount work was carried out on the identification of filamentous bacteria by FISH that are involved in bulking sludge and scum formation (e.g. Erhart et al. 1997, Davenport et al. 2000, Liu and Seviour 2001, Beer et al. 2002, for details see chapter 2.3.2).

2.4.2.5 Comparison of the different molecular biological methods

In the following the pros and contras of the different molecular biological methods are assessed. Cloning technique, RFLP, and DGGE rely on DNA extraction and PCR amplification, which might bias the results by difficulties in sequence retrieval from the environmental samples as described above (chapter 2.4.2.2). In addition, cloning artifacts might misrepresent the gene ratio of the microbial community analyzed within the clone libraries. RFLP is not a reliable tool to report microbial diversity within natural habitats because the number of bands cannot directly be related to the amount of the organisms present in the biocenosis investigated. In contrast to the full-cycle 16S rRNA approach, DGGE is less labor-intensive and hence more suitable to describe complex microbial dynamics, which require the investigation of large amounts of samples. However, DGGE profiles might as well not represent the true diversity of the microbial community due to difficulties in separating the different DNA sequences as is discussed in chapter 2.4.2.3. In addition, DGGE patterns reveal no information about the phylogenetic affiliation and the amount of the different bacteria being present in the environment examined. For this purpose sequence data are necessary. The separation of relatively small DNA fragments of up to 500 bp provides only restricted sequence information. This represents a disadvantage of the DGGE technique especially in the presence of unknown organisms that require in general full-length sequences to reveal an accurate phylogenetic classification (Ludwig et al. 1998). The described obstacles applying cloning, RFLP, and DGGE require further investigation tools, e.g. FISH, to identify the microbial community of environmental habitats. The FISH method is not dependent on DNA extraction and an amplification step, and is able to identify and quantify the presence of specific organisms in situ. Beside the target accessibility limitation, e.g. insufficient cell wall permeability and low ribosome content (Amann et al. 1995), the availability of suitable rRNA-targeted oligonucleotide probes is known to be a demerit of the FISH method. The latter can be solved by applying the full-cycle rRNA approach where the last step includes probe design for unknown organisms followed by applying FISH with these newly designed probes. The cloning approach, followed by sequencing, sequence analysis, probe design and FISH are complementary techniques. The design of FISH probes requires DNA sequence data and the clone libraries provide that. On the other hand the FISH technique is able to prove whether the clone libraries really imply sequences of the microbial community examined.

3 OBJECTIVES

Three factors are known to be responsible for the formation of a stable scum layer: (i) dispersed gas bubbles, (ii) surface-active components and (iii) hydrophobic material (Soddell and Seviour 1990). The hydrophobic and surface-active components in the wastewater might promote scum formation either by direct chemical interaction and/or influencing the growth of a specific bacterial biocenosis. These so-called scum bacteria are known to be able to produce biosurfactants and/or hydrophobic cell surfaces in order to improve access to hydrophobic substances as a carbon source. In addition, extracellular polymeric substances (EPS) of activated sludge and scum might act as sorption sites for both hydrophobic substances and hydrophobic bacteria increasing hydrophobic sludge properties and thus scum flotation. Furthermore these characteristic hydrophobic EPS might provide nutrient sources supporting the growth of hydrophobic organisms. The aim of this thesis is to confirm these hypotheses. Two main topics were investigated: (i) the characterization of the scum bacteria populations and (ii) the physicochemical analysis of activated sludge and scum.

3.1 Characterization of the Scum Bacteria Populations

The characterization of the scum bacteria by classical microscopic sludge analysis comprised the identification of morphotypes that allows comparison with literature data. In addition, the phylogeny of these bacteria reveals information about their physiology, e.g. cell surface properties, biosurfactant production, and the ability to use hydrophobic substances as carbon sources. This approach was divided into two main investigation parts: screening studies and detailed identification of the nocardioform actinomycete group.

3.1.1 Screening of Scum Bacteria Populations

The development of specific scum bacteria populations as described by different work groups all over the world showed *Microthrix parvicella* to be detected most frequently followed by nocardioform actinomycetes. Most of these survey studies describe only morphotypes and do not provide information about the phylogenetic classification in general. Therefore the purpose of this study was to characterize these bacteria on the basis of their morphology and classical staining behavior. In addition, they were identified by fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes specific for filamentous scum bacteria. On the basis of these results the possibilities and limitations of both methods are discussed with focus on the following questions:

- Are the filamentous morphotypes characterized by a single entity or else by various groups, genera, and species?
- Is FISH with specific probes sufficient for phylogenetic documentation of the selected scum bacteria?

3.1.2 Identification of Nocardioform Actinomycetes (*Corynebacterineae*)

Nocardioform actinomycetes are characterized by classical microscopy as Gram-positive branched filaments with two different morphotypes: *Gordonia amarae*-like organisms (GALO) with right angle branching and pine tree-like organisms (PTLO) with acute angle branching. In contrast to the PTLO morphotype that is specific for *Skermania piniformis*, right angle branching of GALO is performed by different genera within the nocardioform actinomycete group (NOC). Furthermore the screening investigations of this study revealed non-branching filamentous morphotype (NOC-like organisms) that was identified by FISH as nocardioform actinomycete but not yet identified on genus or species level. The FISH method with published probes is limited as probes are available for *Skermania piniformis* and the genus *Gordonia*, and *Gordonia amarae* only. As they cover merely a small part of the species diversity within nocardioform actinomycetes different molecular biological methods, the full-cycle rRNA approach and genetic fingerprinting techniques (RFLP and DGGE), were chosen for diversity studies. The aim of this approach was to reveal phylogenetic information on the dominant nocardioform actinomycetes at genus or species level, which might reveal knowledge about physiological properties of these organisms supporting scum formation. Different molecular biological methods should give an answer to the following questions:

- Are both full-cycle rRNA approach and DGGE technique successful tools to identify the nocardioform actinomycetes present in scum samples at genus or species level?
- Are RFLP and DGGE techniques successful methods to organize clone libraries into taxonomic groups and to screen clone libraries for redundancy, and thus to avoid time-consuming work of the full-cycle rRNA approach?

3.2 Bacteria and Physicochemical Analysis of Activated Sludge and Scum

Many research groups analyzed activated sludge EPS and discussed their functions. However, little is known about EPS composition and function in scum. The next aim of this study was thus to correlate the occurrence of dominant scum bacteria identified by quantitative FISH analysis to physicochemical parameters such as EPS quantity and composition, as well as sludge hydrophobicity. Furthermore it was of interest whether the active growth process of scum bacteria takes place in the activated sludge or in the scum layer. These studies are focusing on the following questions:

- Is scum formation a passive floating process of activated sludge to the water surface or an active growth process of a specific biocenosis on the water surface?
- Do EPS provide specific nutrients in activated sludge and/or scum that promote the extensive growth of scum bacteria?
- Do cell surface hydrophobicity and hydrophobic sites of the EPS influence scum development?

4 MATERIAL AND METHODS

4.1 Wastewater Treatment Plants (WWTPs)

4.1.1 WWTPs selected for Screening Investigations

Activated sludge and scum samples were collected from the aeration tanks of 32 municipal WWTPs all over Germany with permanent or intermittent scum problems. The samples were transported (1 to 2 h) to the laboratory for further treatment immediately or sent by mail within 1 day after sampling. Six WWTPs were repeatedly sampled. In those cases where the filamentous bacteria biocenosis remained stable the result was considered as one representative sample. When the biocenosis differed significantly the results were counted as different samples. For each of the six repeatedly sampled WWTPs a shift in the bacterial population was observed twice. Therefore a total of 38 different activated sludge and scum samples were analyzed. Most of the WWTPs were operated as single-stage activated sludge process under low sludge load conditions (F/M ratio < 0.2 kg BOD_5 kg^{-1} MLSS d^{-1}) with nitrogen and phosphorus elimination. In addition, samples were collected from one two-stage activated sludge treatment plant (for details see chapter 4.1.2) and three sequencing batch reactors. More detailed information on the operating parameters is shown in Figure 2.

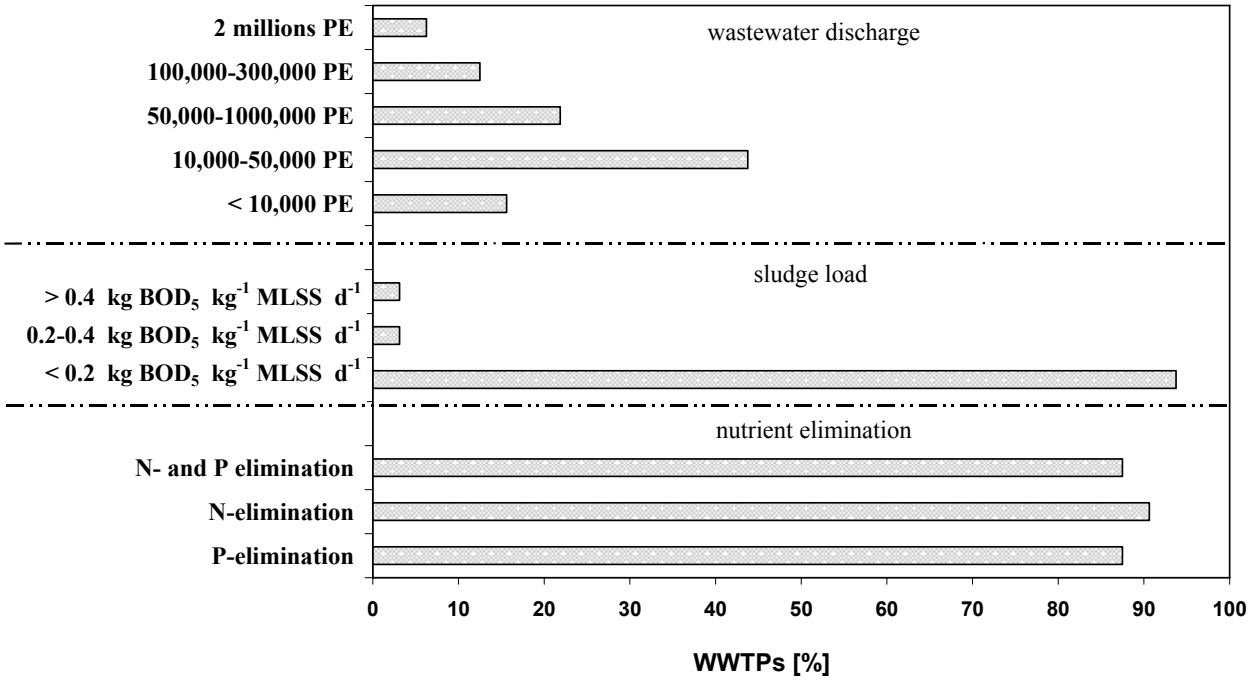


Figure 2: Operating parameters of municipal WWTPs (n=32), where activated sludge and scum samples were collected for screening of scum bacteria populations.

4.1.2 WWTPs selected for Identification of Nocardioform Actinomycetes / Biological and Physicochemical Analyses

The detailed identification of nocardioform actinomycetes was carried out on scum samples collected from five municipal WWTPs dominated by nocardioform actinomycetes growing as different morphotypes. Two WWTPs, WWTP-M and WWTP-BT, were situated in South Germany and the remaining three WWTPs, WWTP-TH, WWTP-OX, and WWTP-IN, were located in East Australia close to the city of Brisbane. For biological and physicochemical analyses the German municipal WWTPs (Bavaria) - WWTP-M, WWTP-BT, and WWTP-GR, characterized by different scum biocenoses - were selected. For this approach activated sludge, scum, and primary effluent from the aeration tank were collected. All samples were transported immediately (1 to 2 h) to the laboratory for further treatment. Detailed operating parameters of these WWTPs are described in the following.

German two-stage WWTP-M
The two-stage activated sludge plant with approximately 2 millions population equivalents (PE) consists of a high load stage for pre-denitrification and C-elimination (aeration tank 1, AT1, F/M ratio 0.64 kg BOD_5 kg^{-1} MLSS d^{-1}) and a low load stage for nitrification (aeration tank 2, AT2, F/M ratio 0.02-0.05 kg BOD_5 kg^{-1} MLSS d^{-1}). Phosphorus is removed by chemical precipitation. During the investigation period scum formation was observed in AT1 only from January 2002 to begin of June 2002 and at the end of November 2003. In contrast, scum developed throughout the year 2002 and during winter and autumn of 2003 in AT2. Only during spring and summer of 2003 there was no scum layer detected on the water surface of AT2. Samples were taken from both tanks, AT1 (denitrification zone) and AT2, between January 2002 until November 2003.

German single-stage WWTPs: WWTP-BT and WWTP-GR
WWTP-BT with 82,000 PE and WWTP-GR with 25,000 PE are single-stage nutrient removal plants (activated sludge process) with intermittent nitrogen elimination and chemical phosphorus removal. The sludge load is below 0.15 kg BOD_5 kg^{-1} MLSS d^{-1}. In both plants a stable brown compact sludge layer was observed on the water surface of the aeration tank during the investigation period. Samples were collected from May 2003 until February 2004 for WWTP-BT and from January 2003 until November 2003 for WWTP-GR.

Australian single-stage WWTPs: WWTP-TH, WWTP-OX, and WWTP-IN
All Australian WWTPs are operated as single-stage activated sludge process. WWTP-OX is characterized by 185,000 PE, whereas the two other plants are much smaller (30,000 PE = WWTP-TH and 17,000 PE = WWTP-IN). Biological nitrogen removal is performed in both WWTP-OX and WWTP-TH, and phosphorus elimination by EBPR technology is only performed in WWTP-TH. In contrast, WWTP-IN consists of an aerobic zone for carbon elimination only. Selective scum control is performed by specific scum harvesters (Fig. 3) in WWTP-OX and WWTP-TH and by chlorinating the return activated sludge (RAS) in WWTP-IN. The scum harvester removes the scum layer on the water surface once per day and reduces the development of stable brown scum layers. Scum samples were collected before scum harvesting from the aeration tank of the WWTP-OX and WWTP-TH in June 2002. No stable scum formation was observed on the water surface of the aeration tank at WWTP-IN. Therefore scum samples were taken from the scum-scraping zone (June 2002) where one-day-old scum, originating from the aeration tank, was stored.

Figure 3: Scum harvester installed on the aeration tank of Australian WWTP-TH.

4.2 Microbial Community Analyses

4.2.1 Microscopic Sludge Analysis of Conspicuous Microorganisms

Filamentous bacteria were identified by classical microscopic sludge analysis described by Eikelboom (2000). This method includes phase contrast microscopy and the examination of Gram- and Neisser-stains by transmission microscopy (Axioplan 2, Zeiss, Jena, Germany). The determination of the individual filamentous organisms was performed using a specific identification key system based on morphology and staining properties.

The filamentous scum bacteria were quantified by (i) classifying dominant and secondary occurrence and (ii) the filament index (FI) according to Eikelboom (2000). The "secondary" filamentous bacteria in general represent 5 to 10% of the total "dominant" filamentous bacteria. The FI method was applied for the quantification of the individual filamentous organisms using class indices from 0 (no filamentous organisms) to 5 (extensive growth of filamentous organisms) where the class index steps differ by a factor of 10.

4.2.2 Fluorescence in situ Hybridization (FISH)

The FISH technique includes different procedures such as cell fixation, pre-treatment of the fixed cells, hybridization, and washing. A detailed description of this method is found in Amann (1995).

4.2.2.1 Sample fixation and pre-treatment to improve cell wall permeabilization

Sample fixation

For all sludge samples one part was fixed in 3% paraformaldehyde, and one part in 50% ethanol. Three volumes of 4% paraformaldehyde (PFA) fixative were added to one volume activated sludge or scum (1:10 diluted in PBS) and incubated at 4°C for 24 h. The fixed samples were centrifuged at 5,000 x g for 5 min and the fixative was removed. The pellet was

washed in PBS, and then resuspended in PBS and absolute ethanol was added to a final concentration of 50%. Ethanol fixation was performed by adding one volume absolute ethanol to one volume activated sludge or scum (1:10 diluted in PBS). No washing step was included for the ethanol fixed samples in order to prevent cell lysis. All fixed samples were stored at –20°C. Before further treatment or hybridization the fixed samples (5 to 10 µl depending on the biomass concentration) were immobilized on microscopic slides (Teflon-coated slides separating 6 or 10 reaction fields, Paul Marienfeld KG, Bad Mergentheim, Germany) by air drying and dehydrating for 3 min in an ethanol series of 50, 80, and 100% (v/v).

1xPBS (Phosphate Buffered Saline)

Na_2HPO_4/NaH_2PO_4	10 mM
NaCl	130 mM
pH 7.2	

3xPBS

Na_2HPO_4/NaH_2PO_4	30 mM
NaCl	390 mM
pH 7.2	

4% PFA Fixative (100 ml)

Paraformaldehyde	4.0 g
H_2O_{bidest}, heated at 60°C	65.0 ml
2M NaOH few drops, until solution becomes clear	
3xPBS	33.0 ml
pH 7.2	
Sterile filtration	

Pre-treatment to improve cell wall permeabilization

The investigation of Gram-positive bacteria in general required ethanol fixation as described above and a further treatment to improve cell wall permeability. The following permeabilization procedures were carried out directly on the reaction fields of the microscopic slides.

a) Enzyme pre-treatment using lysozyme according to Beimfohr et al. (1993)

   The enzyme solution was prepared adding 10 mg lysozyme (Serva, 183,000 U mg^{-1}) to 1 ml lysozyme buffer. Approximately 25 µl lysozyme solution was added to each slide reaction field and incubated for 30 min at room temperature. Afterwards the slides were rinsed with H_2O_{bidest}, air-dried, and dehydrated in an ethanol series as previously described.

   Lysozyme Buffer

Tris/HCl	100 mM
EDTA	50 mM
pH 8	

b) Enzyme pre-treatment using lipase and proteinase K according to Davenport et al. (2000)

The dehydrated cells were incubated at 37°C with 10 µl of a high concentration lipase solution (Sigma, 75 U μl^{-1} in PBS) for 60 min. Slides were placed in a humid chamber containing adsorbent paper wetted with PBS to stop evaporation of the enzyme solution. The lipase solution was removed after incubation by immersing the slide in H_2O_{bidest}. The slide was air-dried before treatment with proteinase K. The cells were incubated at 37°C for 30 min with proteinase K (Sigma, final concentration 10 µg ml^{-1}), then rinsed with H_2O_{bidest}, air-dried, and dehydrated as previously described.

c) Mild acid hydrolysis pre-treatment according to Macnaughton et al. (1994)

The microscopic slides spotted with dehydrated cells were subjected to hydrochloric acid (1M HCl) at 37°C for 30 min followed by a washing step with H_2O_{bidest}, air-drying, and dehydration with ethanol.

d) Combination of acid hydrolysis and enzyme treatment

The dehydrated cells were treated first with hydrochloric acid as described in (c), followed by treatment with lysozyme as described in (a), lipase/proteinase K as described in (b) or by applying lysozyme and lipase/proteinase K following protocol (a) and (b).

4.2.2.2 Hybridization procedure and counterstaining with 4´,6-Diamidino-2-Phenylindole (DAPI)

FISH was performed according to Amann (1995) with minor modifications. The hybridization buffer (8 µl) was spread on each slide reaction field and the remainder was used to provide a moisture chamber to prevent evaporation of the hybridization buffer. Afterwards 1 µl of fluorescent rRNA-targeted oligonucleotide probe (30 ng μl^{-1}) was added and mixed carefully. The slides were transferred to a moisture chamber and hybridized at 46°C for at least 2 h. For the identification of the Gram-positive bacteria the recommended hybridization time of 2 h was increased to 15 h to enhance probe penetration. After the incubation time the slides were removed from the moisture chamber and the hybridization was immediately stopped by rinsing the probe from the slides with a pre-warmed washing buffer (48°C). Afterwards the slides were transferred into the washing buffer and incubated at 48°C for 20 min. Salts were removed by shortly dipping the slides in H_2O_{bidest}. Slides were air-dried.

DAPI staining was performed by applying approximately 20 µl of a DAPI solution to each reaction field containing the hybridized cells. Samples were incubated at 4°C for 30 min; the DAPI solution was removed by rinsing with H_2O_{bidest}. Slides were air-dried.

The oligonucleotide probes used in this study are described in Table 2 with corresponding specificity, morphotype according to Eikelboom (2000), and the recommended formamide concentration. Detailed information about probe sequences and target sites are listed in the appendix 9.1, Table 20. All probes were purchased from MWG Biotech (Ebersberg, Germany). Probes targeting specific taxa were labeled with sulfoindocyanine dye Cy3 (indocarbocyanine) and probes for the domain *Bacteria* (EUB338-mix = EUB338, EUB338-

II, and EUB338-III) were labeled with sulfoindocyanine dye Cy5 (indodicarbocyanine) or FLUOS (5,(6)-carboxyfluorescein-N- hydroxysuccinimide ester).

Table 2: rRNA-targeted oligonucleotide probes with corresponding specificity, morphotype according to Eikelboom (2000), and recommended formamide (FA) concentration.

oligonucleotide probe	specificity[a]	morphotype	FA [%]	reference
EUB338 EUB338-II EUB338-III = EUB338-mix	*Bacteria* *Planctomycetes* *Verrucomicrobia*		0-60	(Amann et al. 1990) (Daims et al. 1999)
ACA23a	*Acinetobacter* spp.	type 1863	35	(Wagner et al. 1994)
HGC69a	*Actinobacteria*		25	(Roller et al. 1994)
HGC1156	*Actinobacteria*		20	(Erhart 1997)
HGC235 HGC270H	*Actinobacteria* helper probe for HGC235		10 10	(Erhart 1997) (Glöckner et al. 2000)
MPA60 MPA223 MPA645 = MPA-mix	"*Candidatus* Microthrix parvicella" unclassified *Actinobacteria*	*Microthrix parvicella*	20	(Erhart et al. 1997)
MNP1	nocardioform actinomycetes = *Mycolata* = *Corynebacterineae*	GALO[b]	50	(Schuppler et al. 1998)
Myc657	nocardioform actinomycetes = *Mycolata* = *Corynebacterineae*	GALO PTLO[c]	30	(Davenport et al. 2000)
CMN119	subgroup of nocardioform actinomycetes without: *Gordonia* spp., *Skermania piniformis*, *Dietzia* spp., *Tsukamurella* spp.	GALO	30	(Erhart 1997)
G-Gor-0596-a-A22	*Gordonia* spp.	GALO	20	(de los Reyes et al. 1997)
NPI425	*Skermania piniformis*	PTLO	20	(Bradford 1997)
NLIMI 91	*N. limicola* I isolates, *Trichococcus* spp. within the *Firmicutes* phylum	*Nostocoida limicola* I	20	(Liu and Seviour 2001)
NLIMII 175 NLII65	"*Candidatus* Nostocoida limicola" nearest relative *Tetrasphaera japonica* within the *Actinobacteria* phylum	*Nostocoida limicola* II	20	(Liu and Seviour 2001) (Bradford 1997)
AHW183	*N. limicola*-like isolate nearest relative *Sphaerobacter thermophilus* within the *Chloroflexi* phylum	*Nostocoida limicola* II	35	(Schade et al. 2002)

Table 2 continued.

oligonucleotide probe	specificy[a]	morphotype	FA [%]	reference
PPx3-1428	"*Candidatus* Alysiomicrobium bavaricum"	*Nostocoida limicola* II	50	(Snaidr et al. 2002, Levantesi et al. 2004)
Noli-644	"*Candidatus* Alysiosphaera europaea"		35	
MC2-649	"*Candidatus* Monilibacter batavus"		35	
	nearest relative *Rhodospirillum* sp. within the *Alphaproteobacteria* class			
NLIMIII 301	*N. limicola* III isolate, *Isosphaera pallida* within the *Planctomycetes* phylum	*Nostocoida limicola* III	20	(Liu and Seviour 2001)
0092-997	type 0092 isolate	type 0092	20-25	(Bradford 1997)
	nearest relative *Flavobacterium columnare* within the *Bacteroidetes* phylum			
CHL 1851	type 1851 isolate	type 1851	20	(Beer et al. 2002)
	nearest relative *Roseiflexus castenholzii* within the *Chloroflexi* phylum			
TM7905	nearly entire candidate division TM7	type 0041/0675	20	(Hugenholtz et al. 2001)
TM7305	subdivision 1 of candidate division TM7		30	

[a] bacterial taxonomy and nomenclature according to Garrity et al. (2004)
[b] GALO = *Gordonia amarae*-like organisms, [c] PTLO = pine tree-like organisms

Hybridization Buffer

Formamide	x[a] µl
5 M NaCl	360 µl
1 M Tris/HCl, pH 8	40 µl
10% sodium dodecylsulfate (SDS, w/v)	2 µl

H_2O_{bidest} add up to a final volume of 2 ml

[a] Formamide concentration depending on the required stringency, which differs for the various probes used, see Table 2.

Washing Buffer

1M Tris/HCl, pH 8.0	1000 µl
5 M NaCl	x[a] µl
0.5 M EDTA, pH 8.0	x[b] µl
10% sodium dodecylsulfate (SDS, w/v)	50 µl

H_2O_{bidest} to a final volume of 50 ml

[a] Stringency of the washing buffer is increased by lowering the sodium chloride concentration, see Table 3.
[b] 500 µl 0.5 M EDTA was added for 20% formamide concentration and above.

4´,6-Diamidino-2-Phenylindole (DAPI) solution
DAPI dissolved in H_2O_{bidest}, final concentration 1 µg ml^{-1}.

Table 3: Sodium chloride content in the washing buffer depending on the formamide concentration used in the hybridization buffer.

formamide [%]	NaCl [mol]	5 M NaCl [µl]
0	0,900	9000
5	0,636	6300
10	0,450	4500
15	0,318	3180
20	0,225	2150
25	0,159	1490
30	0,112	1020
35	0,08	700
40	0,056	460
45	0,04	300
50	0,028	180

4.2.3 Determination of Metabolic Activity

Dehydrogenase enzymes are present in all microorganisms and consequently dehydrogenase activity is used as an indicator for metabolic activity. In this study the respiratory microbial activity of activated sludge and scum biocenoses was analyzed by determining the dehydrogenase activity using the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polyscience Inc., Eppelheim, Germany). The detection of respiratory activity using CTC is based on the reduction of water-soluble and colorless tetrazolium salts to colored crystals of the water-insoluble formazan (CTF) products. These formazan crystals can be detected by spectrophotometry after extraction of the formazan crystals using ethanol or at the single cell level by epifluorescence microscopy or confocal laser scanning microscopy (CLSM). Both methods were performed according to Griebe et al. (1997) with slight modifications.

4.2.3.1 Spectrophotometric analysis of formazan crystals (CTF)

Overall metabolic activity was measured by incubating 3 g activated sludge or scum (1:10 diluted in PBS) with 5 mM CTC at 25°C for 3 h in the dark. The suspension was stirred to maintain aerobic conditions during incubation. Afterwards the samples were centrifuged at 12,000 x g for 3 min and the supernatant was removed. The pellets were resuspended in 100% ethanol and sonicated for 2 min. Both the application of ethanol and sonication treatment cause cell lysis and extraction of the CTC-formazan crystals. After this procedure the samples were centrifuged at 12,000 x g for 3 min, the supernatant was collected, and the pellets were resuspended for a second time with ethanol and treated with sonication. This procedure -

centrifugation, collecting of the supernatants, adding ethanol, and sonication - was repeated until the supernatant became colorless and consequently all CTC-formazan crystals were extracted. The absorbance of the collected supernatants was determined at 450 nm. The produced formazan (CTF) concentration was calculated according to the Lambert-Beer equation using the molar excitation coefficient of CTF; ε = 20200 (Severin and Seidler 1998). The redox activity was calculated as CTF concentration per mass volatile solids (VS) and time, given in μmol g^{-1} h^{-1}.

4.2.3.2 In situ detection of formazan crystals (CTF) at single cell level

Incubation with 1 ml of activated sludge or scum (diluted 1:10 in PBS) and 5 mM CTC was carried out as described above. The reduction of CTC was stopped by adding paraformaldehyde (final concentration 3%) and incubation for 30 min. Afterwards paraformaldehyde was removed by centrifugation at 5,000 x g for 10 min and the pellets were resuspended in PBS. Approximately 10 µl sample volume was applied to a microscopic slide, dried, and counterstained with DAPI as described in chapter 4.2.2.2. Qualitative detection of CTF-positive cells was performed using CLSM with the specific laser for fluorescence dyes CTF and DAPI (see the following chapter).

4.2.4 Microscopy and Digital Image Analysis

After in situ hybridization or CTC staining the microscopic slides were embedded in Citifluor AF1 (Citifluor, Canterbury, Great Britain) to prevent bleaching of the fluorescent signals. Samples were analyzed by (i) epifluorescence microscopy or (ii) confocal laser scanning microscopy (CLSM).

Epifluorescence microscopy and quantification of FISH signals

The epifluorescence microscope (Axioplan 2, Zeiss) was equipped with 10x/0.30 Ph1, 40x/1.30 Ph3, 63x/1.30 Ph3 and 100x/1.30 Ph3 plan-neofluar objectives with oil immersion for the 40x, 63x and 100x objectives. The individual filter sets (AHFanalysentechnik, Tübingen, Germany) that were used for the specific fluorescence dyes are listed in Table 4. In addition, the microscope was supplied with filters specific for phase contrast microscopy. Microscopic images were recorded by a digital camera (AxioCam Color, Zeiss) and the software AxioVision (Zeiss).

Table 4: Epifluorescence microscope, features of the used filter sets.

filter set AHF-nr.	**excitation filter**	**beam splitter**	**emission filter**	**fluorescence dye**
F11-000	D 360/40	400 DCLP	GG 420	DAPI
F44-001	HQ 480/40	Q 505 LP	HQ 510 LP	FLUOS formazan (CTF)
F41-007	HQ 545/30	Q 505 LP	HQ 610/75	CY3
F41-008	HQ 620/60	Q 660 LP	HQ 700/75	CY5, SYTO 60

Epifluorescence microscopy was used to quantify FISH signals from the screening investigations applying the class index to compare quantitative data with the classical microscopic sludge analysis. The class indices of 0 to 5 were evaluated by qualitative description of the amount of the detected organisms compared to quantitative data determined as percentage of specific organisms of total bacteria (Table 5). The class index steps differ approximately by the factor of 10. Biomass concentrations needed to be comparable on all slides examined in order to obtain correct quantitative data, which was checked by DAPI staining or by applying the EUB338-mix probes specific for the domain *Bacteria*.

Table 5: Class indices of 0 to 5 used for the quantification of FISH signals.

class index	qualitative description of the amount of detected organisms	quantification [%]
0	none	0
1	few	<1
2	some	1-10
3	frequent	10-20
4	many	20-40
5	excessive	>40

Confocal laser scanning microscopy (CLSM) and quantification of FISH signals
All confocal images were recorded using an LSM 510 (Zeiss) including an Axioplan 2 as described above. The CLSM was equipped with two internal helium-neon lasers with 543 nm and 633 nm, one external argon laser with 488 nm, and a UV laser with 364 nm. The individual lasers with their dye specificity and corresponding emission filter are shown in Table 6.

Table 6: CLSM, lasers with dye specificity, and the corresponding emission filter.

laser	emission filter	fluorescence dye
UV, 364 nm	LP 385	DAPI
external argon laser, 488 nm	LP 505	FLUOS formazan (CTF)
internal helium-neon laser, 543 nm	LP 560	CY3
internal helium-neon laser, 633 nm	LP 650	CY5, SYTO 60

For quantification of the FISH signals including the recording of images and microscope set-up the protocol of Daims et al. (2001b) was followed. For this approach the sludge was prepared on microscopic slides comprising 10 reaction fields and the spotting (10 µl of fixed sample) and drying procedures were repeated at least three times to obtain a thick layer of sludge on the slide surface. The thick sludge layer is required to detect as many target cells as

possible to improve the accuracy of the quantitative data. Two internal helium-neon lasers with 633 nm and 543 nm were used as the excitation source for the Cy5-labeled EUB338-mix probes and the Cy3-labeled probes specific for different bacteria groups, genera, or species, respectively. For each quantification 15 to 30 microscopic fields were scanned as single images using a 63x oil immersion objective. Images were stored as RGB TIFF images and analyzed with the software Image Pro Plus (Media Cybernetics, Gleichen, Germany) to measure the pixel area of the CY3 and the CY5 signals. The area fraction for the specifically stained cells was calculated as a percentage of the total area of *Bacteria* stained by the EUB338-mix probes in the same image.

area fraction of specific probe to EUB338-mix [%] = 100 * (pixel area of the CY3 and CY5) / (pixel area of CY5)

4.2.5 DNA Extraction

DNA was extracted from scum samples (originating from WWTP-M, AT1 and AT2, WWTP-BT, and WWTP-TH) and pure cultures with Gram-positive cell walls using the FASTDNA SPIN Kit for soil (BIO 101, Qbiogene, Heidelberg, Germany). Sample processing was carried out as suggested by the manufacturer using 200 µl scum samples or approximately two inoculation loops of a pure culture growing on agar plates. The different extraction steps include cell lysis, purification, enrichment, and elution of the isolated DNA. The extracted DNA was visualized by agarose gel electrophoresis and stored in ultra pure H_2O (DNase/Pyrogen free water) at –20°C.

4.2.6 Polymerase Chain Reaction (PCR)

4.2.6.1 PCR technique

The amplification of rDNA fragments was performed by the PCR technique using the HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany). The reaction mixture for PCR was prepared as followed:

<u>100 µl PCR Reaction Mixture</u>

- HotstarTaq Master Mix 50 µl
  0.02 U μl^{-1} HotStarTaq polymerase
  0.2 mM (each) dATP, dCTP, dGTP and dTTP
  1.5 mM $MgCl_2$
- each primer (200 ng μl^{-1}) 1 µl
- DNA template x µl[a]
- H_2O_{bidest} add up to a final volume of 100 µl

[a] 1 to 2 µl of cell suspension (clones) or approximately 100 ng of extracted DNA

The reaction mixture was placed in a thermal mastercycler (Eppendorf, Hamburg, Germany). After that a specific cycling program was applied (see Table 7).

Table 7: Cycling program

step	time [min]	T [°C]	number of cycles
initial activation	15	95	1
denaturation	0.5	94	1
annealing	0.5	variable (see Table 8)	30
extension	2	72	1
final extension	10	72	1

PCR primers

The PCR primers used were synthesized by MWG Biotech (Ebersberg, Germany). Table 8 shows the primers with respective specificity, sequence, annealing temperature, and reference. For most of these primers the annealing temperature was applied as recommended in reference, with the exception of the *Actinobacteria*-specific primer pair Act235f and AB1156r, where the annealing temperature was evaluated using specific pure cultures for target and non-target organisms (see Table 9).

Table 8: Primers for amplification of 16S rDNA and plasmid primers for amplification of clone inserts.

primer	specificity	sequence (5´-3´)[a]	T_a [°C][b]	reference
27f	*Bacteria*	AGAGTTTGATCMTGGCTCAG	48	(Lane 1991)
1492r	*Bacteria*	TACGGYTACCTTGTTACGACTT	48	(Lane 1991)
342f	*Bacteria*	CTCCTACGGGAGGCAGCAG	55	(Lane 1991)
518r	*Bacteria*	GWATTACCGCGGCKGCTG	55	(Lane 1991)
Act235f	*Actinobacteria*	CGCGGCCTATCAGCTTGTTG	58	(Stach et al. 2003)
AB1165r	*Actinobacteria*	ACCTTCCTCCGAGTTRAC	58	(Lüdemann and Conrad 2000)
SP6	plasmid	ATTTAGGTGACACTATAGAA	48	
T7	plasmid	TAATACGACTCACTATAGGG	48	

[a] using oligonucleotide IUB-codes
[b] T_a = annealing temperature

Table 9: Pure cultures used for evaluation of the annealing temperature for *Actinobacteria*-specific primers Act235f and AB1165r with the respective growth conditions (medium and temperature) and DSM numbers (DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany).

species	source	medium	T [°C]
Comamonas testosteroni	DSM 50244	R2A[a]	28
Ralstonia eutropha	DSM 531	R2A	28
Gordonia amarae	DSM 43392	R2A	28
Rhodococcus fascians	DSM 20669	R2A	28
Rhodococcus globerulus	DSM 43954	R2A	28
Rhodococcus rhodnii	DSM 43336	R2A	28
Rhodococcus ruber	DSM 43338	R2A	28

[a] detailed description of R2A medium see appendix 9.2

Visualization of PCR amplicons and DNA quantification
A 2% TAE agarose gel was loaded with 3 µl PCR product and 3 µl loading buffer (5x BlueRun, MWG Biotech). Electrophoresis was performed in a horizontal gel-electrophoresis system with 1x TAE as the running buffer at a constant voltage of 100 V for 1 to 1.5 h. After electrophoresis, the gel was incubated for 20 min in H_2O_{dest} containing ethidium bromide (1 µg ml^{-1}) and afterwards rinsed for 15 min in H_2O_{dest}. The detection of the DNA bands was carried out with the gel documentation system GelSystem Compact including UV transilluminator (302 nm), CCD camera and Poretix software (biostep, Jahnsdorf, Germany). The DNA amount was estimated by comparing the fluorescence intensity of DNA bands with the corresponding band size of a 100 bp DNA ladder (peQLab, Biotechnologie, Erlangen, Germany), which was applied at a defined concentration to each electrophoresis run.

1x TAE Buffer

Tris base	40 mM
Acetic acid	20 mM
EDTA, pH 8	1 mM

Purification of PCR amplicons for cloning and sequencing
PCR amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer protocols and eluted in H_2O_{bidest}. After the purification step the PCR amplicons were once more checked by horizontal agarose gel electrophoresis.

4.2.6.2 Application of the PCR technique

For the following PCR applications the PCR conditions (PCR reaction mixture and cycling program) described above were used with the exception of the nested PCR applied before DGGE analysis where a different cycling program was used (see Table 10).

The PCR technique was applied using *Actinobacteria*-specific primers Act235f and AB1165r to prove whether the extracted DNA included *Actinobacteria* species. The organisms within the phylum *Actinobacteria* are characterized by a very complex cell wall and consequently cell lysis is hampered for the DNA isolation.

Amplification of the near-complete 16S rRNA genes from extracted DNA (MAT1, MAT2, BT, and TH) was achieved by employing the bacterial conserved 16S rRNA primers 27f and 1492r in a PCR. These amplicons were purified and immediately used for cloning (see chapter 4.2.7). Moreover PCR was applied for screening the clone inserts using *Actinobacteria*-specific primers (Act235f and Ab1165r) and for amplification of the complete clone inserts using plasmid primers SP6 and T7 that were used for RFLP analysis (see chapter 4.2.8) and sequencing (see chapter 4.2.10).

DGGE analysis (see chapter 4.2.9) required DNA fragments smaller than 200 bp because experience of this study showed that longer fragments cause problems with band separation. Therefore nested PCR technique was applied before DGGE analysis. Clone inserts were amplified first with plasmid primers T7 and SP6, and afterwards reamplified with nested primers 342f and 518r. *Actinobacteria*-specific primers Act235f and AB1165 were used to amplify environmental DNA (MAT1, MAT2, and BT) followed by reamplification with the nested primers 342f and 518r. The nested primer 342f used has at its 5´-end an additional 40-nucleotide GC-rich sequence (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G, GC clamp, Muyzer et al. 1997) to improve resolution of the DNA fragments in the denaturing gradient. For the nested primers 342f and 518r a specific cycling program (see Table 10) was used in order to obtain optimal DGGE results.

Table 10: Cycling program for PCR with the nested primers 342f and 518r used to generate amplicons for DGGE analysis.

step	time [min]	T [°C]	number of cycles
initial activation	15	95	1
denaturation	1	94	1
annealing	1	55	30
extension	1	72	1
final extension	30	72	1

4.2.7 Cloning of Near-Complete 16S rRNA Genes (Clone Libraries)

The pGEM^{R}-T Easy Vector System including competent cells (Promega, Madison, USA) was used for cloning of purified PCR amplicons containing near-complete 16S rRNA genes. The cloning was performed according to the manufacturer protocol following the different procedures: ligation, transformation, cultivation of transformed cells, and storage of transformed cells. In this study 4 different 16S rDNA clone libraries (TH, MAT1, MAT2, and

BT) were produced from PCR amplicons of extracted DNA originating from scum samples of WWTP-TH, WWTP-M, AT1 and AT2, and WWTP-BT.

The ligation was carried out by incubation of purified PCR amplicons with the pGEM^R-T Easy Vector in a ligation reaction mixture at 4°C overnight. After incubation the ligation reaction mixture was briefly centrifuged. Two µl of the reaction mixture was added to a 1.5 ml microcentrifuge tube on ice. Frozen competent cells (*E. coli*, JM109, stored at –80°C) were thawed on ice. A volume of 70 µl of thawed cells was gently placed in the 1.5 ml tube containing 2 µl ligation reaction mixture as described above, gently mixed and incubated on ice for 20 min. After a heat-shock treatment for 45 to 50 sec in a water bath at exactly 42°C, the tube was immediately returned to ice for further 2 min. 950 µl of SOC medium (appendix 9.2) was added to the tubes containing the transformed cells. Tubes were shaken at 37°C for 1.5 h. A volume of 100 µl of transformed cells was spread onto Luria-Bertani agar plates (LB medium, appendix 9.2) that contained 100 mg L^{-1} ampicillin and were supplemented with 20 µl of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 50 mg ml^{-1}) and 100 µl of 100 mM IPGT (isopropyl-β-D-thiogalactopyranoside solution). Both X-Gal and IPGT were spread over the plate surface and adsorbed at 37°C for 30 min prior to use. Inoculated agar plates were incubated at 37°C overnight (16 to 24 h) and afterwards at 4°C for 2 h to increase blue color intensity facilitating blue/white screening. Positive clones (those containing 16S rDNA PCR inserts) appeared white and negative clones (no inserts) were blue. Positive clones were picked, homogenized into 100 µl of sterile 20% glycerol, and stored at –20°C. At least 100 clones were stored for each clone library.

Ligation Reaction Mixture

- 2x rapid ligation buffer 5 µl
- pGEM^R-T Easy Vector (50 ng) 1 µl
- T4 DNA ligase (3 Weiss units $µl^{-1}$) 1 µl
- PCR product (75 ng) 2 µl
- H_2O_{bidest} 1 µl

4.2.8 Restriction Fragment Length Polymorphism (RFLP) Analysis of Clone Inserts

The PCR amplicons (plasmid primer pair T7 and SP6) from the individual clones were subjected to restriction enzyme Hae III (Sigma) digestion. Hae III is a restriction enzyme that recognizes and cuts tetranucleotide sequences 5´GG-CC3´ ("4-bp cutter"). The digestion mixture (see below) was incubated at 37°C overnight (16 h). The restriction-digested fragments were separated by horizontal electrophoresis in a 3% TAE agarose gel, loaded with 10 µl reaction mixture and 5 µl loading buffer, for 1.5 h at 80 V. RFLP patterns were visualized after ethidium bromide staining described above and photographs of the gel were taken using the gel documentation system GelSystem Compact (see above, 4.2.6.1). Clones containing inserts that produced identical restriction patterns were grouped.

Digestion Mixture

- NEB buffer (Sigma) 4 µl
- Hae III enzyme (10 U $µl^{-1}$) 1 µl
- PCR amplicon 22 µl
- H_2O_{bidest} 7 µl

4.2.9 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed with the Bio-Rad DCode system (BioRad, Hercules, USA). An 8% polyacrylamide gel with gradients was formed with 8% acrylamide stock solutions containing 50% and 70% denaturants (100% denaturant contains 7 M urea and 40% formamide, see below for the preparation of stock solutions) according to the manufacturer instructions. Twenty µl nested PCR product of environmental DNA mixed with 10 µl loading buffer and 4 µl nested PCR product of clone inserts mixed with 4 µl loading buffer were applied to the acrylamide gel. Electrophoresis was performed in 1x TAE buffer at 70 V and at 60°C for 16 h. After electrophoresis the gels were stained for 30 min in 1x TAE containing SYBR Gold (1: 1000; Molecular Probes, Eugene, OR, USA), and afterwards rinsed briefly in H_2O_{bidest}. The DGGE bands were detected and documented by the gel documentation system described in chapter 4.2.6.1 using a specific emission filter (537 nm) for the CCD camera. DGGE bands of interest were excised from the gel and DNA was eluted in sterile H_2O_{bidest} at 37°C overnight. Eluted DNA was amplified, purified, and subsequently sequenced.

50% Denaturing Solution of an 8% (w/v) Polyacrylamide Gel (100 ml)

40% Acrylamide/Bisacrylamide (37:1)	20.0 ml
50x TAE buffer	2.0 ml
Formamide	20.0 ml
Urea	21.0 g
H_2O_{bidest}	58.0 ml

70% Denaturing Solution of an 8% (w/v) Polyacrylamide Gel (100 ml)

40% Acrylamide/Bisacrylamide (37:1)	20.0 ml
50x TAE buffer	2.0 ml
Formamide	28.0 ml
Urea	29.4 g
H_2O_{bidest}	50.0 ml

50x TAE Buffer

Tris base	2 M
Acetic acid	1 M
EDTA, pH 8	50 mM

4.2.10 Sequencing and Analysis of Sequence Data

Purified PCR amplicons of clone inserts (plasmid primer pair SP6 and T7) and of eluted DNA from DGGE bands (primer 342f and 518r) were sequenced by MWG-Biotech. The recommended DNA amount (20 ng DNA/100 bases), for clone inserts approximately 300 ng and for eluted DNA from DGGE bands approximately 40 ng, was dried in a SpeedVak centrifuge and sent by mail to the company. The primer 342f was employed to obtain sequence data from the DGGE bands and partial sequence data from clone inserts. In addition, primer SP6, T7, 518r, and 926f (Table 11) were used to determine near-complete sequence information from clone inserts.

Table 11: Primers used for sequencing.

primer	specificity/target site	sequence (5´-3´)[a]
SP6	plasmid	ATTTAGGTGACACTATAGAA
T7	plasmid	TAATACGACTCACTATAGGG
342f	*Bacteria*, 16S rDNA	CTCCTACGGGAGGCAGCAG
518r	*Bacteria*, 16S rDNA	GWATTACCGCGGCKGCTG
926f	*Bacteria*, 16S rDNA	AAACTYAAAKGAATTGACGG

[a] using oligonucleotide IUB-codes

The partial 16S rDNA sequences were compared with those in public databases by using the program Basic Local Alignment Search Tool (BLAST, Altschul et al. 1990, NCBI, http://www.ncbi.nlm.nih.gov/). Partial and full-length 16S rDNA sequences were analyzed to be chimeric sequences using the CHIMERA CHECK software tool of the Ribosomal Database project (RDP II, Cole et al. 2003, http://rdp.cme.msu.edu). Full-length 16S rDNA sequences were analyzed by the ARB software package (Ludwig et al. 2004) including sequence alignment, similarity sequence analysis, and reconstruction of phylogenetic trees of the new sequences and related organisms. Tree topologies were evaluated by neighbor joining, maximum parsimony, and maximum likelihood analyses in combination with different filters excluding highly variable positions. A consensus tree was constructed with topology corrected by consideration of the results of the various tree reconstruction algorithms. Before phylogenetic analysis was performed the ARB 16S rRNA sequence database was updated with sequences of related organisms to the full-length sequences of this study originating from public databases detected by the BLAST tool. The similarity matrix tools of the the RDP II and of the ARB software was applied to determine sequence similarity of environmental DNA DGGE bands to the corresponding clone inserts and related organisms.

4.2.11 Design of 16S rRNA-targeted Oligonucleotide Probes and Evaluation

Specific probes for species within the nocardioform actinomycete group were designed using the probe design tool in the ARB software package. Based on comparative analysis of all sequences in the ARB database comprised of publicly available sequences and of full-length 16S rDNA sequences of this study, the program selected specific regions within the groups or species sequences of interest that allows their discrimination from all other reference sequences. Oligonucleotide probe sequences were subsequently confirmed for specificity using the probe match tool in the ARB software and additionally BLAST, which has access to databases containing all publicly available sequences. The designed oligonucleotides (see Table 18 chapter 5.2.3.6) were synthesized and labeled with sulfoindocyanine dye CY3 by MWG-Biotech.

The newly designed probes were evaluated with ethanol fixed pure cultures or scum samples. The formamide concentration for optimum probe stringency was determined by performing a series of FISH experiments at 10% formamide increments starting at 0% formamide up to

50% formamide. Fluorescence signal intensity of the oligonucleotide probes applied on pure culture or scum sample using different formamide concentrations was checked qualitatively by epifluorescence microscopy. The selection of the pure cultures of target organisms (no mismatch with the probes sequences) and non-target organisms (with the fewest mismatches to probe sequences) was performed using the probe match tool of the ARB program. The pure cultures were ordered from DSMZ, cultured under recommended growth conditions (Table 12), and fixed in 50% ethanol.

Table 12: Pure cultures used for probe evaluation with DSM numbers and recommended growth conditions (DSMZ).

species	source	medium[a]	T [°C]
Dietzia maris	DSM 43672	65	28
Gordonia aichiensis	DSM 43978	65	28
Gordonia sputi	DSM 43896	65	37
Mycobacterium celatum	DSM 44243	354	37
Mycobacterium phlei	DSM 43239	65	37
Nocardia corynebacteroides	DSM 20151	53	30
Rhodococcus erythropolis	DSM 43188	65	28

[a] detailed description of the various media see appendix 9.2

4.3 Physicochemical Analyses

4.3.1 Determination of Volatile Solids (VS)

The determination of VS was carried out following the protocol of DIN 38414 S2 and S3 (DEV 1998). 80 g dispersed activated sludge or 30 g scum was filled into crucibles and dried at 105°C till constant weight (= total solid). Afterwards the ignite residue was determined by heating the samples at 550°C for 60 min in a muffle furnace. VS was calculated by subtracting the amount of ignite residue from the one of total solid.

4.3.2 Extraction of Extracellular Polymeric Substances (EPS)

EPS were extracted from activated sludge and scum by cation exchange resin (CER) using a DOWEX 50 x 8, 20-50 mesh in the sodium form (Fluka) following Frølund et al. (1996). The method is based on removing cations from the sludge matrix leading to a breakup of the flocs and a subsequent release of EPS. Many authors compared different physical and chemical extraction techniques reviewed by Nielsen and Jahn (1999). Extraction using CER has been reported to be the most successful method in terms of EPS yield and minimal disruption of the exopolymers and microbial cells.

In order to remove any EPS from bulk water a washing step was performed on activated sludge. Dispersed activated sludge (500 g) was centrifuged at 10,000 x g for 15 min at 4°C. The supernatant (H_2O-phase) was stored for further analysis at –20°C. The sludge pellets were resuspended in approximately 100 ml extraction buffer followed by a second

centrifugation step. Afterwards sludge pellets and 25 g scum without an intermittent washing step were suspended in 20 ml extraction buffer and homogenized for 10 min with a Teflon piston (1500 rpm) to improve extraction efficiency. The EPS extraction was performed as follows: Homogenized activated sludge and scum samples were transferred to an extraction beaker with baffles and filled up with 480 ml extraction buffer (500 ml final volume of extraction buffer). 100 g CER (washed for 1 h in extraction buffer prior to use, CER amount should be approximately 65-80 g g^{-1} VS) was added to each sample. The suspension was stirred at 4°C for 3 h in the dark. The extracted EPS were harvested by centrifugation of the CER/sludge suspension at 12,000 x g for 10 min at 4°C. The supernatant (EPS) was stored at – 20°C for further analysis. Cellular lysis due to the extraction procedure was examined by performing glucose-6-phosphate dehydrogenase (G-6-PDH) activity measurements in the EPS extract using the G-6-PDH kit from Sigma (Catalog No. 345).

Extraction Buffer

Na_2PO_4 * 12 H_2O	2 mM
NaH_2PO_4 * H_2O	4 mM
NaCl	9 mM
KCl	1 mM
pH 7	

4.3.3 Analysis of Primary Effluent, Water Phase, and EPS Extract

Primary effluent, the sludge supernatant (H_2O-phase), and the EPS from activated sludge and scum were analyzed with respect of total organic carbon (TOC), lipophilic substances, carbohydrate, and protein. Parameters are determined as mg L^{-1} (primary effluent, H_2O-phase) and mass EPS per mass volatile solids (VS), given as mg g^{-1} (EPS components). Total carbohydrate and protein content were calculated in TOC units using the factor 0.375 for protein (reference substance = bovine serum albumin, BSA) and the factor 0.378 for carbohydrate (reference substance = glucose).

TOC measurements

The determination of TOC was performed according to DIN 38409 H3 (DEV 1998) using a TOC analyzer (Total Carbon Monitor 480, CE Instruments, Great Britain). The samples were injected into the TOC analyzer where they were acidified with 0.5% HNO_3 and purged of inorganic carbon. Organic carbon was oxidized by nickel oxide at 1020°C. The produced CO_2 was reduced quantitatively to methane by copper with hydrogen at 420°C. Methane was detected using a flame ionization detector (FID).

Analysis of lipophilic substances

Lipophilic substances were measured by infrared spectroscopy following DIN 38409 H18 (DEV 1998) with some modifications. The extraction of lipophilic substances was carried out by mixing the samples with the solvent frigen for 10 min, followed by phase separation. The organic phase was analyzed by infrared spectroscopy detecting the amount of hydrocarbons. The concentration of lipophilic substances was calculated using different group excitation coefficients for CH_3-, CH_2- and CH-bands that are empirically determined and described in DIN 38409 H18.

Determination of protein
The determination of protein was performed by a modified method according to Lowry (1951) with BSA as standard. One-milliliter sample was mixed with 1.4 ml reagent 4 and incubated for 20 min at room temperature. Reagent 5 was added (0.2 ml) and the solution was mixed. After 30 min incubation at room temperature the absorbance at 750 nm was measured by spectrophotometry.

Reagent 1
NaOH 143 mM
Na_2CO_3 270 mM

Reagent 2
$CuSO_4$ 57 mM

Reagent 3
Na-tartrate 124 mM

Reagent 4 was made up using reagents 1 to 3 in the proportion 100:1:1 (freshly prepared prior to use).

Reagent 5
Folin-Ciocalteu-Phenol reagent (Merck) diluted 5:6 with H_2O_{dest}

Determination of carbohydrate
The anthrone method (Dreywood 1946) was applied for carbohydrate determination with glucose as standard. One-milliliter sample was mixed with 2 ml 75% H_2SO_4 followed by the addition of 4 ml anthrone reagent (0.2% anthrone (w/v) in 75% H_2SO_4) and incubated at 100°C for 10 min in a water bath. After cooling of the samples the absorbance was measured at 578 nm by spectrophotometry.

4.3.4 Microbial Adhesion To Hydrocarbons (MATH)

Hydrophobicity of activated sludge and scum before and after EPS extraction was determined by microbial adhesion to hydrocarbons (MATH) following Rosenberg et al. (1980). Samples were centrifuged at 12,000 x g for 15 min and the pellets were resuspended in PUM (Phosphate-Urea-Magnesium) buffer (sludge pellets after EPS extraction were directly resuspended in PUM buffer) and a second centrifugation step was carried out. The pellets were again resuspended in PUM buffer and an optical density (436 nm) of 0.7 to 0.8 was adjusted. One-milliliter hexadecane was added to 5 ml sample suspension and in parallel a control reaction without hexadecane was prepared. All reactions were incubated at 30°C for 15 min followed by 2 min Vortex mixing and 15 min incubation at room temperature to allow hexadecane separation. The absorbance at 400 nm of the water phase was measured in the reaction tube with hexadecane ($A_{hexadecane}$) and in the reaction tube without hexadecane ($A_{without\ hexadecane}$) by spectrophotometry. Results were calculated as the percentage of the hydrocarbon-adsorbed sludge fraction as followed:

$$\text{Hydrophobicity [\%]} = 100 - (A_{hexadecane}/A_{without\ hexadecane})*100$$

PUM Buffer (Phosphate-Urea-Magnesium Buffer)

$K_2HPO_4 * 3H_2O$	100.0 mM
KH_2PO_4	50.0 mM
Urea	30.0 mM
$MgSO_4 * 7H_2O$	0.8 mM
pH 7	

4.3.5 Microsphere Adhesion to Cells (MAC)

The MAC technique was applied to determine in situ hydrophobic properties of the sludge samples especially the cell surfaces of scum bacteria. The method was performed according to Nielsen et al. (2001) with some modifications. Hydrophobic yellow-green fluorescent microspheres (FluoSpheres, diameter of 0.2 µm, Molecular Probes) consisting of polystyrene and with sulfate-modified surface were applied. The excitation/emission wavelengths were 505/515 nm. The microspheres were diluted in H_2O_{dest} to an 0.2% (w/v) solution and sonicated to obtain a uniformly dispersed solution (sonication for 10 min at 60 W). Activated sludge and scum (diluted 1:10) were washed in PBS by centrifugation at 5,000 x g for 5 min and the pellets were resuspended in 1 or 2 ml PBS depending on the biomass concentration. Part of the samples was stained with the nucleic acid dye SYTO 60 (Molecular Probes) by incubating 200 µl sample with 1 µl SYTO 60 solution (5 mM) for 30 min, followed by a washing step to remove the non-bound dye. 200 µl of stained and non-stained samples were mixed with 20 µl 0.2% microspheres solution, incubated at 30°C for 5 min, and vigorously shaken on a Vortex mixer for 2 min. An aliquot of 5 to 10 µl of the sample was transferred onto microscopic slides and dried. Gram staining procedure was carried out afterwards on samples without SYTO 60. The samples can be stored at 4°C for some hours before examination.The microscopic slides were embedded in Citifluor AF1 prior to use and investigated by transmission/epifluorescene microscopy (Gram staining/microspheres) and CLSM (SYTO 60/microspheres).

5 RESULTS

5.1 Screening of Scum Bacteria Populations

It is well known that filamentous bacteria occur often in high numbers during scum events (for details see chapter 2.3.2). Figure 4 shows stable gas bubbles of a scum sample (A). At a higher magnification filamentous bacteria associated with the surface of these bubbles were visualized (Fig. 4 B). For that reason the microscopic sludge analysis of filamentous populations according to Eikelboom (2000) was used to describe the bacteria biocenosis occurring in scum. Many of these morphologically distinguishable scum bacteria are so-called morphotypes and for most of these bacteria only recently progress in resolving their systematic has been made. For many of these organisms rRNA-targeted oligonucleotide probes are available (Table 2 lists the probes applied with references, chapter 4.2.2.2). These probes were applied for FISH to demonstrate whether the morphotypes belong phylogenetically to one or to various groups, genera, or species.

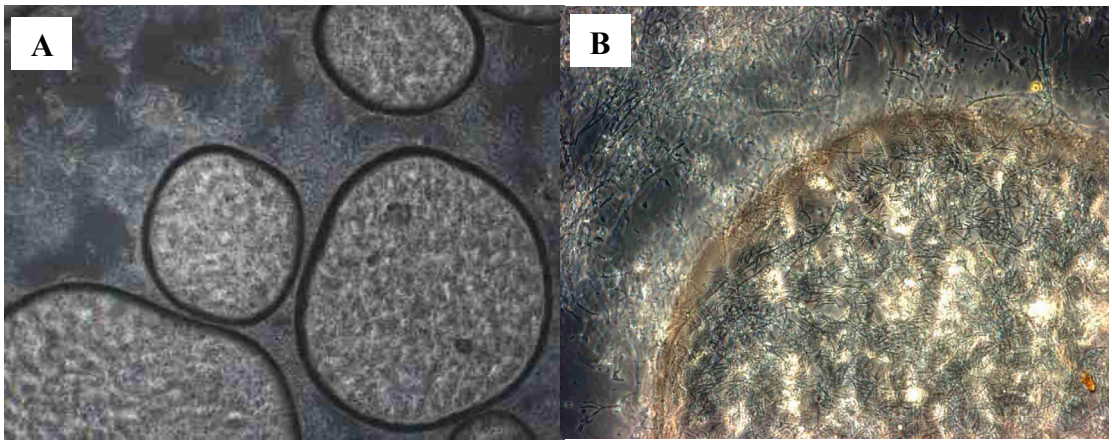


Figure 4: Phase contrast images of a scum sample showing stable gas bubbles (**A**, 100x) with associated filamentous bacteria (**B**, 400x).

The main focus of the screening studies presented here was to record the occurrence of specific scum bacteria and to quantify their abundance in activated sludge and scum samples by both classical microscopic sludge analysis and FISH. A rough quantification was performed by using the terms dominant and secondary to characterize overall abundance of filamentous bacteria by classical microscopy according to Eikelboom (2000). In addition, the class index system (0 to 5) was used to get more quantitative information. In activated sludge all detected filamentous organisms were identified, in the scum fraction only the dominant and the secondary organisms with a class index of 2 to 3 were considered.

5.1.1 Microscopic Sludge Analysis of Conspicuous Microorganisms

Screening of the biocenoses (Fig. 5) from 32 municipal WWTPs with permanent or intermittent scum problems revealed that a variety of organisms occurred in activated sludge. Only some of these organisms were found in higher numbers in the scum fraction.

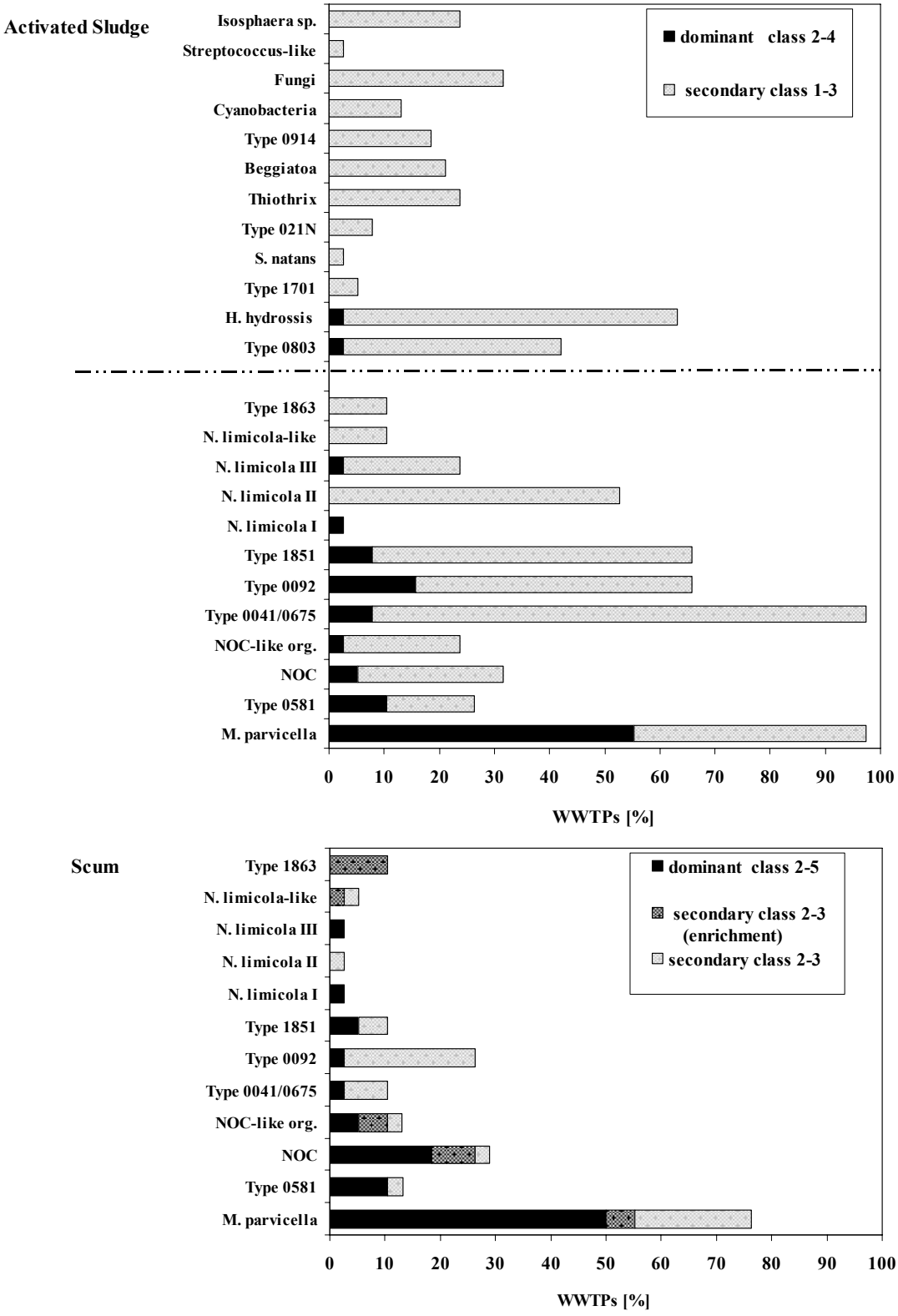


Figure 5: Populations of filamentous microorganisms in activated sludge and scum from WWTPs with scum events (n=38). In activated sludge all detected filamentous organisms were identified, in the scum fraction only the dominant and the secondary organisms with a class index of 2 to 3 were characterized. NOC = nocardioform actinomycetes, Gram-positive branched filamentous bacteria; NOC-like org. = Gram-positive bent rods growing as short non-branched filaments or in cell clusters.

The most frequently identified filamentous organism within 97% of the analyzed activated sludge samples was *Microthrix parvicella*. However, only in about 50% of the examined plants, *M. parvicella* was the dominant filament in both activated sludge and scum. Gram-positive branched filamentous bacteria identified as nocardioform actinomycetes (NOC, GALO = *Gordonia amarae*-like organisms) and Gram-positive bent rods growing as short non-branched filaments or in cell clusters (NOC-like organisms) were detected in 55% of the activated sludge samples but were only characterized in 5% and 3% of the samples as the dominant filamentous bacteria, respectively. In contrast to these observations, NOC and NOC-like organisms were identified in 18% and 5% of the WWTPs as the dominant filamentous organisms in the scum fraction, respectively. A further filamentous organism found enriched in the scum samples was type 1863, detected in 11% of the plants but never occurring as a dominant bacterium.

The filamentous type 0581 showing a high similarity to the morphology of *M. parvicella* was found in 11% of the WWTPs as the dominant filamentous bacterium in both activated sludge and scum. It was remarkable that type 0581 always occurred along with *M. parvicella* and type 0092. Type 0092 was present in 16% of the plants as the dominant filamentous organism in the activated sludge and only in 3% of the plants in the scum layer. The sampling date indicated that type 0092 appeared in higher numbers in summer and autumn, and was detected in much lower abundance during wintertime. The filamentous bacteria types 0041/0675 and 1851 occurred in 97% and 66% of the WWTPs, respectively, but were identified in less than 10% of the WWTPs as the dominant organism in activated sludge and were never enriched in the scum fraction. *N. limicola* with *N. limicola* II as the most frequently detected bacteria (53% of the WWTPs) were found in similarly low numbers.

5.1.2 Comparing FISH with Classical Microscopic Sludge Analysis

The **morphotype *M. parvicella*** (Fig. 6 A) detected in 97% of the WWTPs by classical microscopy (CM) was found at the same frequency after application of FISH using different oligonucleotide probes (MPA60, MPA223, and MPA645 used as MPA-mix) targeting different 16S rRNA regions of the organism "*Candidatus* Microthrix parvicella" (Fig. 7). In one single plant *M. parvicella* filaments (class index of 4) were detected together with single rods (class index of 4) by FISH in both activated sludge and scum. Best FISH signals visualizing the whole filament were obtained after lysozyme pre-treatment and increasing the recommended hybridization time from 2 h (detecting merely fragments of the filaments, Fig. 6 B) up to 15 h (detecting the whole filament, Fig. 6 C). Enzymatic treatment and longer incubation time enhance probe penetration that is often a problem when applying FISH to detect Gram-positive bacteria.

Quantification using the class index (0 to 5) showed that both methods, classical sludge analysis and FISH, yielded similar quantitative data (Fig. 7). In activated sludge, *M. parvicella* was identified in about 86% (CM) and 92% (FISH) of WWTPs within a class index range from 1 to 3 and found in noticeably fewer plants with an index of 4 (11% of WWTPs by CM and 6% of the WWTPs by FISH). In comparison to activated sludge, *M. parvicella* was present in a similar number of plants in scum samples with a class index of 1 and 2. However, a shift from class index 3 to higher values of 4 and 5 was observed in the scum samples. The interpretation of both methods demonstrated a consensus of the

quantification results in 70% of activated sludge and in 44% of scum samples. The highest variation of the quantification data of both methods showed an index difference of 1 with mostly higher class indices for the classical microscopic sludge analysis. *M. parvicella* might be overestimated by the classical method because after the Gram staining in the presence of high numbers of *M. parvicella* other, particularly Gram-negative organisms, are less visible. In contrast to this observation, the application of *Bacteria*-specific probes and DAPI staining clearly visualizes all bacterial organisms by FISH.

The results shown demonstrate that in all plants examined the morphotype *M. parvicella* appears to be one single phylogenetic entity based on its 16S rRNA gene sequence, identified as "*Candidatus* M. parvicella", an unclassified member of the *Actinobacteria* first described by Blackall et al. (1994b). However, *M. parvicella* could not be detected using the *Actinobacteria*-specific probe HGC69a. This makes sense because the 23S rRNA sequence of *M. parvicella* has at least two mismatches with the 23S rDNA probe sequence (Bradford et al. 1998). In contrast to the HGC69a probe, the *Actinobacteria*-specific probe HGC1156 showed clear positive FISH signals.

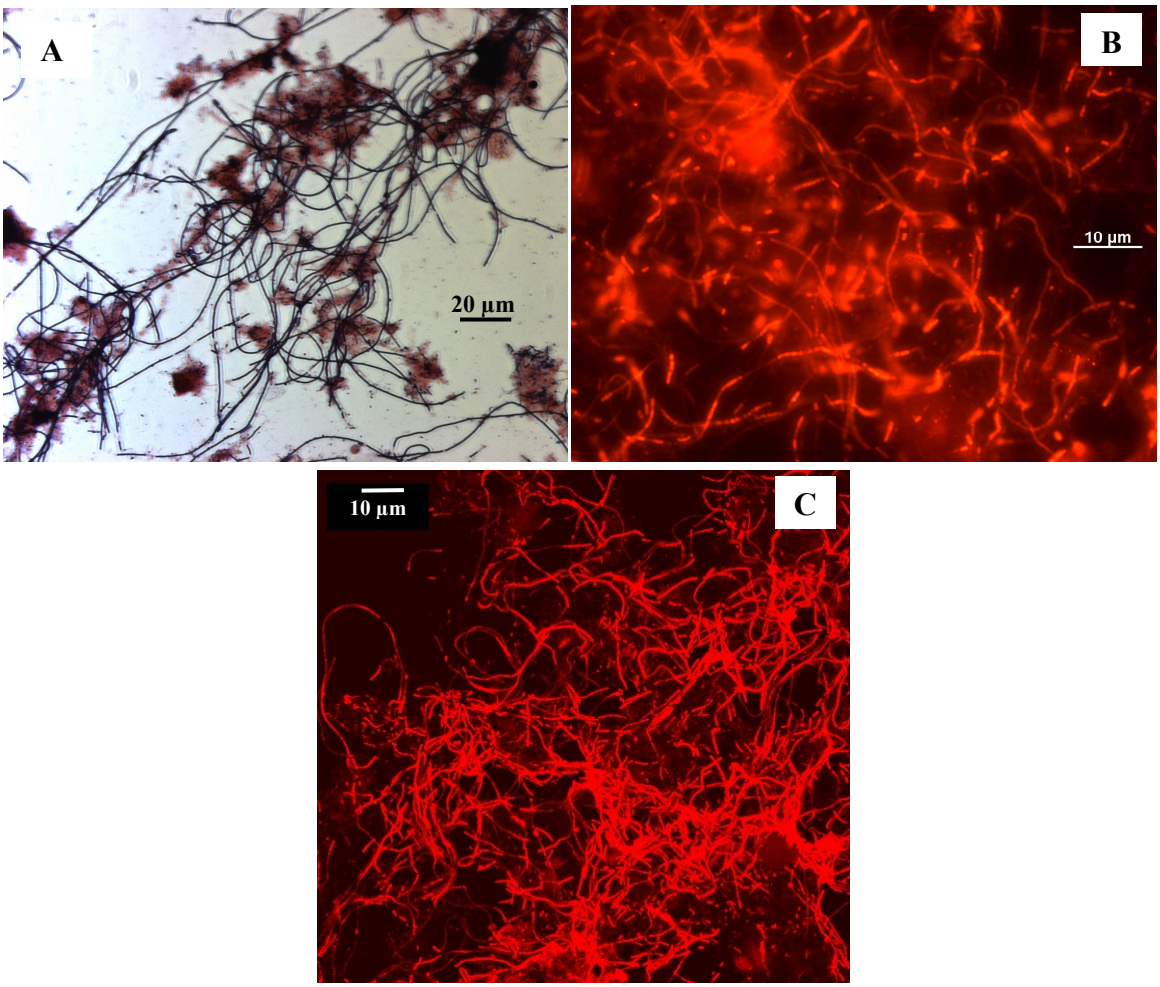


Figure 6: Detection of *M. parvicella* in the scum fraction. Bright field images after Gram staining (**A**). CLSM images after FISH with CY3-labeled MPA-mix (MPA60, MPA223, MPA645) after 2 h hybridization time (**B**) and 15 h hybridization time (**C**).

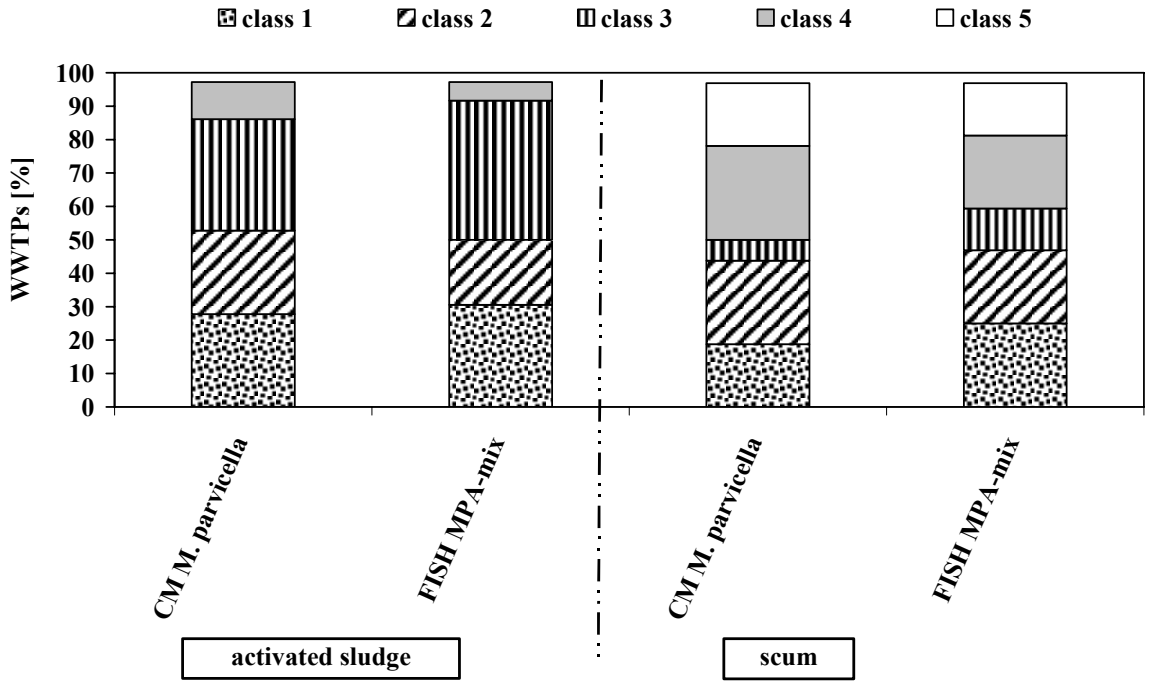


Figure 7: Detection of *M. parvicella* by classical microscopic sludge analysis (CM) and FISH (MPA-mix = MPA60, MPA223, MPA645), activated sludge n=36, scum n=32.

Because of a high morphological similarity of **type 0581** to *M. parvicella* FISH with *M. parvicella*-specific probes was applied to confirm a possible phylogenetic relationship. However, the identification of type 0581 as "*Candidatus* M. parvicella" could only be confirmed for some type 0581 filaments. Type 0581, which were mostly detected along with *M. parvicella*, can easily be distinguished from morphotype *M. parvicella* after Gram and Neisser staining. In contrast, the application of FISH using "*Candidatus* M. parvicella"-specific probes was not suitable to soundly differentiate between both morphotypes, 0581 and *M. parvicella.* Hence no quantification analysis for type 0581 was carried out.

Both **morphotypes, NOC** (Gram-positive branched filaments, GALO = *Gordonia amarae*-like organisms) and **NOC-like organisms** (Gram-positive non-branched short filaments and cell clusters), were identified as nocardioform actinomycetes by FISH after lysozyme pre-treatment and 15 h hybridization time using group-specific probes (MNP1 and Myc657). Both probes are described to detect nearly the entire group of nocardioform actinomycetes (Schuppler et al. 1998, Davenport et al. 2000). The software program BLAST (Altschul et al. 1990) and the probe match program RDP II (Cole et al. 2003) showed both probes to identify the same organisms. However, also different species were targeted. Therefore both probes were analyzed separately. The conspicuous NOC and NOC-like organisms, both identified by FISH as nocardioform actinomycetes, were summarized. In addition to the conspicuous morphotypes, single rods were visible after the application of FISH using the specific probes MNP1 and Myc657. Therefore the FISH results were differentiated in filamentous bacteria (NOC and NOC-like organisms) and non-filamentous bacteria (rods).

In 68% of the activated sludge samples and in 61% of the scum samples NOC (GALO) and NOC-like organisms were found by classical microscopy with a significantly higher detection rate as compared to FISH (Fig. 8). The probe Myc657 turned out to be more successful than

the probe MNP1. The quantitative analysis showed a shift to lower values after FISH in comparison with classical microscopy, particularly after the application of the probe MNP1. This finding was most distinctive at higher classical microscopic ratings of 3 to 5. By both methods a significantly higher number of nocardioform actinomycetes was determined in the scum layer as compared to activated sludge. Furthermore it was observed that probe MNP1 detected NOC-like organisms and failed more frequently to visualize branched filamentous bacteria. Single rod nocardioform actinomycetes were detected in 28 to 64% of sludge samples at a class index of 1 to 2 and in 45 to 70% of scum samples at an index of 1 to 3 by FISH with also a higher detection rate for the Myc657 probe. They often occurred along with filamentous forms but in 14% of the activated sludge samples and in 27% of the scum samples only single rod nocardioform actinomycetes were detected.

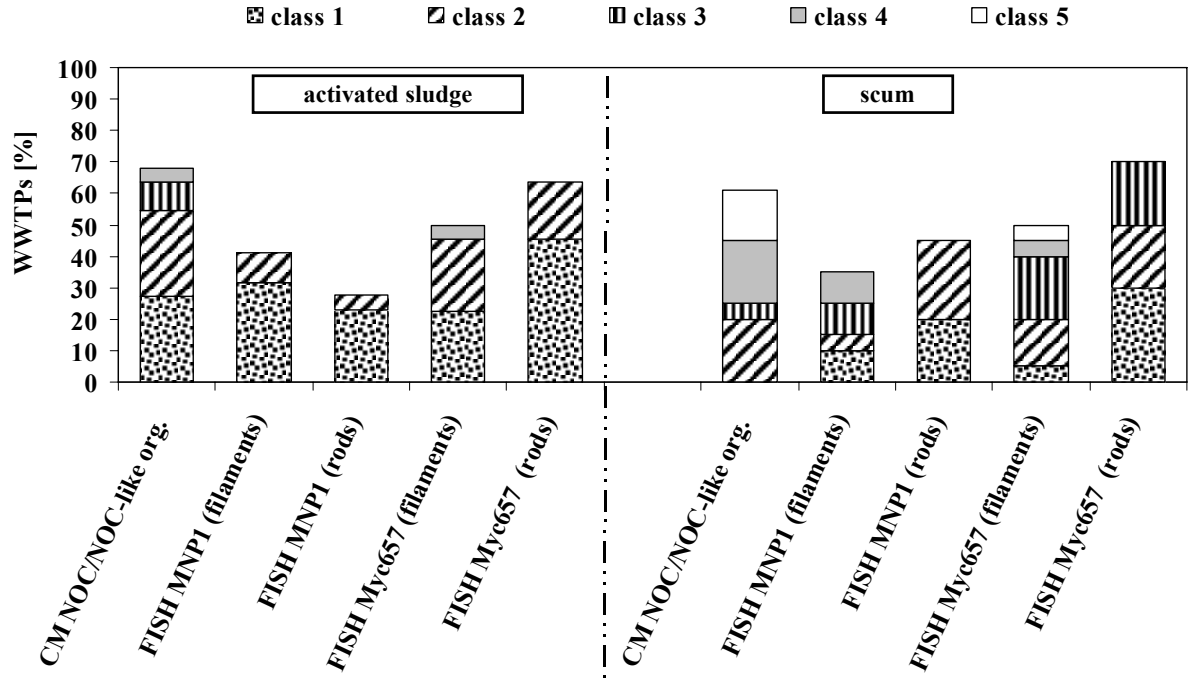


Figure 8: Detection of nocardioform actinomycetes (NOC/NOC-like organisms) by classical microscopic sludge analysis (CM) and FISH (MNP1 and Myc657), activated sludge n=22, scum n=20.

The application of the *Gordonia*–specific probe (Gor596) and S*kermania piniformis*–specific probe (NPI425) gave rare signals only. Consequently no information was obtained on the genus and species level. *Actinobacteria*-specific probes HGC69a and HGC1156 detected most of the nocardioform actinomycetes in the analyzed samples. Some branched filamentous morphotypes did not give any positive FISH signal after application of these general *Actinobacteria*–specific probes. These organisms were neither visualized by group-specific nor by *Bacteria*-specific (applied as EUB338-mix) probes. These results demonstrate that despite enzymatic pre-treatment and longer hybridization time some of the nocardioform actinomycetes were not detectable by FISH. This might explain the lower detection rate by FISH as compared with classical microscopy. The very complex cell wall of this group with its hydrophobic mycolic acids might be the reason for the cell wall to be highly impermeable to oligonucleotide probes. Different protocols to improve permeability are described in the

literature (Macnaughton et al. 1994, Erhart et al. 1997, Davenport et al. 2000, Carr et al. 2005) and were carried out to optimize the FISH signals for selected samples. These results are shown in chapter 5.2 "Identification of Nocardioform Actinomycetes" with findings described in detail. Moreover low or no FISH signals might be attributed to insufficient availability of the target sites, the rRNA molecules, due to low ribosome content (Amann et al. 1995).

Gram-negative type 1863 filaments found in this study together with **Gram-negative coccoid cells** were identified as ***Acinetobacter* spp**. by FISH using the ACA23a probe (Fig. 9). Both morphotypes were clearly enriched in the scum fraction (Fig. 10).

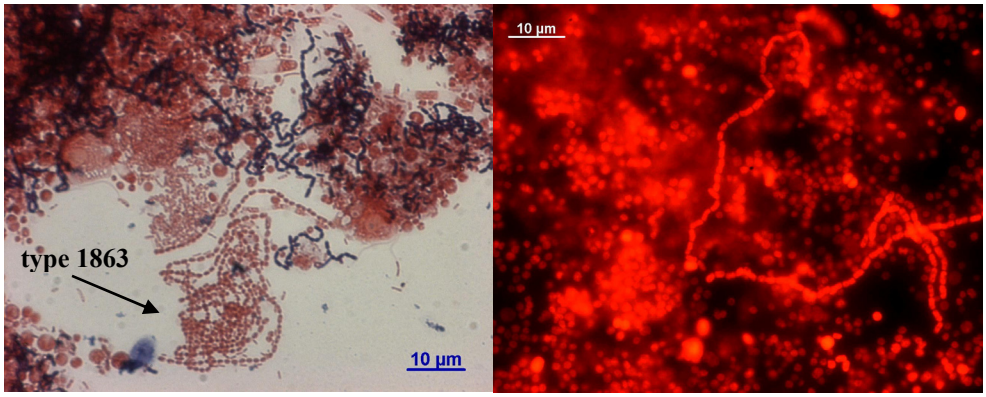


Figure 9: Scum fraction of a high load stage. The bright field image shows filamentous Gram-negative bacteria characterized as type 1863 and Gram-negative coccoid cells. The epifluorescence image confirms *Acinetobacter* spp. after FISH with CY3-labeled ACA23a probe.

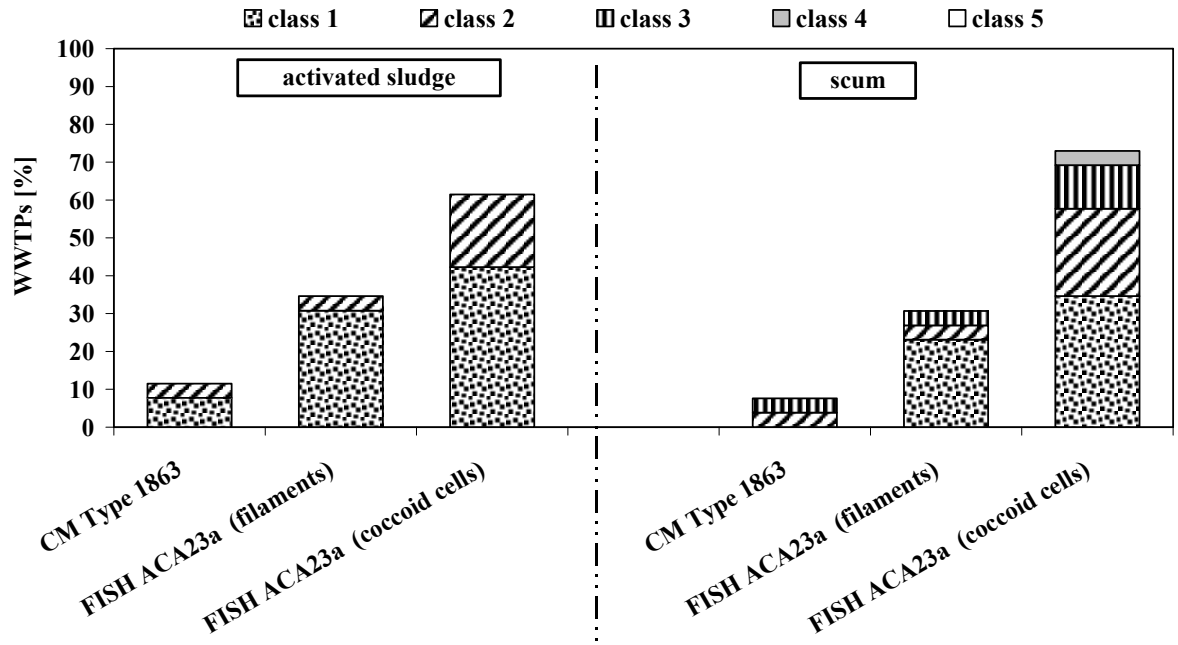


Figure 10: Detection of type 1863 by classical microscopic sludge analysis (CM) and FISH (ACA23a = *Acinetobacter* spp.), activated sludge n=26, scum n=26.

Filamentous *Acinetobacter* spp. were found in 12% and 8% of the activated sludge and scum samples by classical microscopy, respectively. A higher detection rate of approximately 30% was determined after the application of FISH using the ACA23a probe. With regard to quantitative data, both methods detected filamentous *Acinetobacter* in similar numbers of plants at higher class indices of 2 to 3, and classical microscopy underestimated them at lower values (class index of 1). The FISH technique turned out to be the more sensitive method at a low abundance of type 1863. Furthermore only FISH visualized and identified the coccoid cells occurring in many plants at high numbers particularly in the scum fraction.

Most of **the Gram-positive sheathed types 0041/0675 filaments** (Fig. 11 A1 and A2; Fig. 12) gave good signals with the probe TM7905 targeting nearly the entire candidate division TM7, a division exclusively characterized by environmental 16S rDNA sequence data (Hugenholtz et al. 1998). Using transmission electron microscopy, Hugenholz et al. (2001) showed that the sheathed filamentous morphotype 0041/0675 classified within the TM7 division had a typical Gram-positive cell envelope ultrastructure. Despite this Gram-positive feature in these investigations type 0041/0675 was detected by FISH after PFA-fixation and without any enzymatic pre-treatment. Comparing classical methods and FISH using the TM7905 probe similar results with a slightly higher detection rate for the classical method were found for the occurrence of type 0041/0675 (97% by CM and 86% by FISH using TM7905) and the quantitative ranking (class index of 1 to 3). However, FISH often detected only a part of the 0041/0675 morphotypes and quantification using class indices was not sensitive enough to report these differences. The application of the probe TM7305 (Fig. 11 B1 and B2; Fig. 12) detecting TM7 subdivision 1 was less successful. In 30% of the analyzed activated sludge samples type 0041/0675 was visualized with both probes, however, in half of these samples a higher class index was revealed for probe TM7905. Other filaments morphologically identified as type 0041/0675 (12% of the WWTPs) appear to belong to a different phylogenetic bacteria group because these filaments could not be detected by TM7-specific but by *Bacteria*–specific (EUB338-mix) probes. In addition, in 12% of the samples analyzed TM7905 positive filaments were found that did not show the morphological features of type 0041/0675.

The identification of the **type 1851 filaments** (Fig. 11 C1 and C2; Fig. 12) using the CHL 1851 probe demonstrated that these filamentous bacteria fell within the *Chloroflexi* phylum (subdivision 3) closely related to *Roseiflexus castenholzii* (Beer et al. 2002). The FISH results correspond quite well with classical findings. The quantitative analyses showed that type 1851 was found with a class index of 1 to 3 by both methods but a shift to a higher class index value was mostly observed after the application of FISH.

None of the **bacteria type 0092 filaments** (Fig. 12) detected by classical microscopy in activated sludge of 54% of the WWTPs showed positive signals with the probe 0092-997 targeting the 16S rRNA sequence of a type 0092 isolate within the *Flavobacterium* subgroup (Bradford et al. 1996). The application of EUB338-mix probes did not reveal clear detection results for morphotype 0092 either. This morphotype, which is easily recognizable after Neisser staining, could hardly be distinguished from the FISH signals of other filamentous bacteria and hence not clearly be assigned to 0092 filaments by FISH.

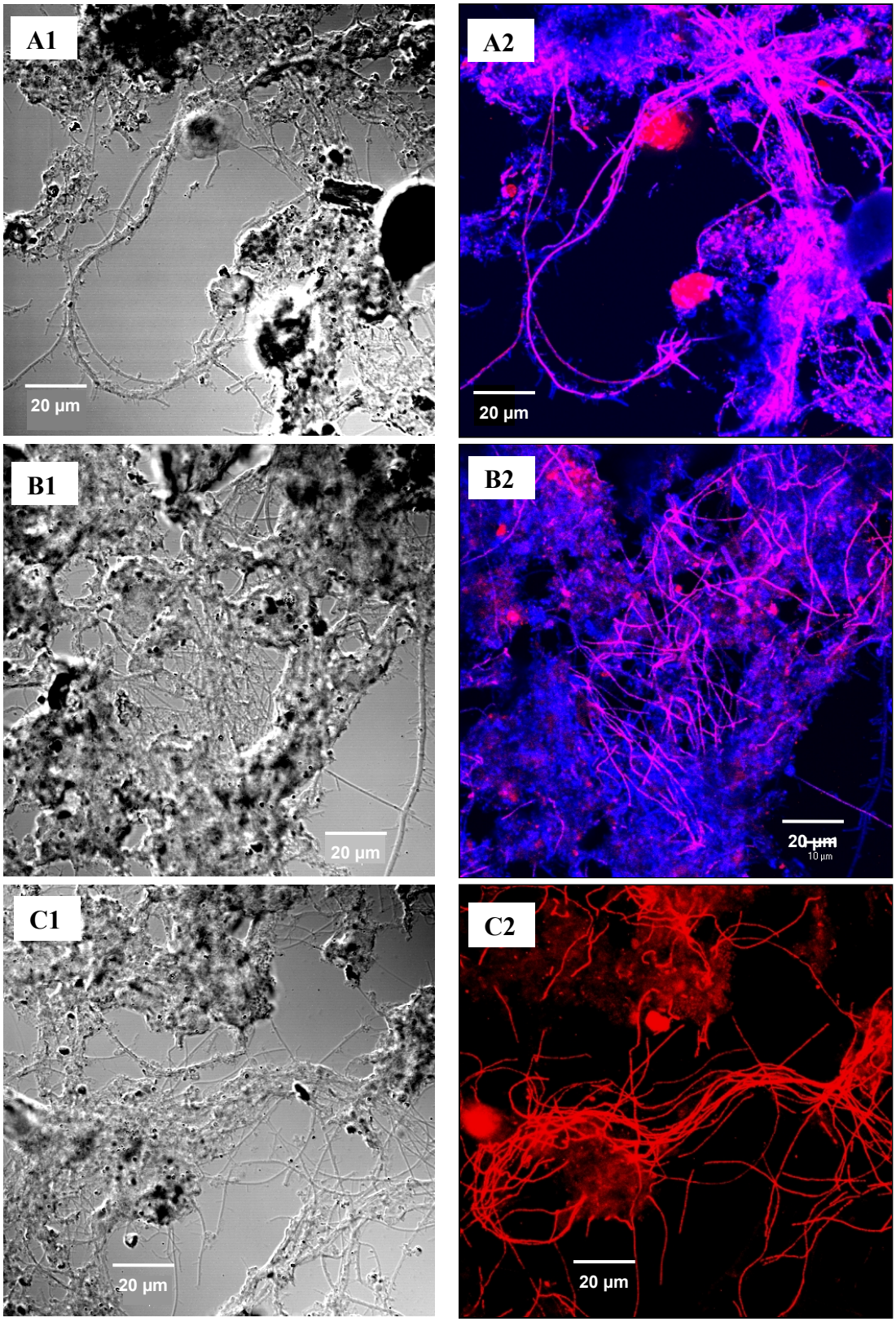


Figure 11: Filamentous **types 0041/0675** and **1851** found in activated sludge samples. Phase contrast images (left side) show filamentous **type 0041/0675** (**A1** and **B1**) and **type 1851** (**C1**). CLSM images (right side) after simultaneous hybridization with CY5-labeled probes EUB388-mix (blue) and CY3-labeled probe TM7905 (red) detecting candidate division TM7 (shown as magenta, **A2**), and CY3-labeled probe TM7305 (red) detecting subdivision 1 of candidate TM7 (shown as magenta, **B2**); after FISH using CY3-labeled probe CHL 1851 identifying type 1851 within the *Chloroflexi* phylum (C2).

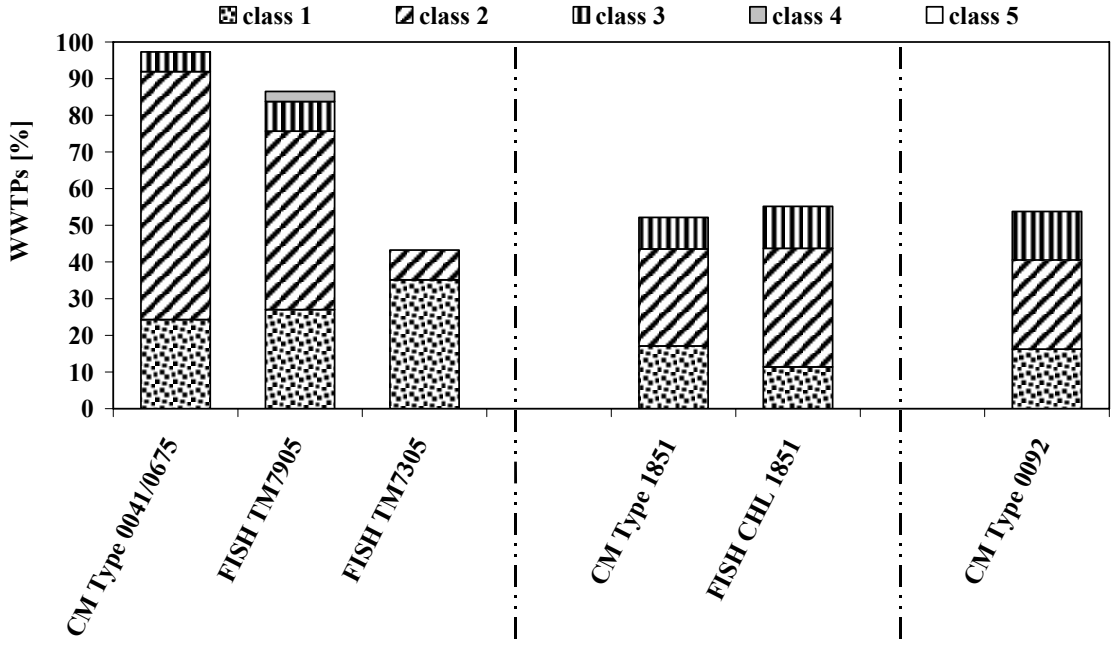


Figure 12: Detection of type 0041/0675 (n = 37), type 1851(n = 34) and type 0092 (n = 37) by classical microscopic sludge analysis (CM) and FISH in activated sludge (probes applied: TM7905 = the entire candidate TM7 division, TM7305 = subdivision 1 of TM7, and CHL 1851 = type 1851 within the *Chloroflexi* phylum).

N. limicola characterized by three different morphotypes by classical microscopy constitutes the most diverse phylogenetic group within the filaments investigated. Different oligonucleotide probes targeting various organisms belonging to different phylogenetic phyla such as *Firmicutes*, *Chloroflexi*, *Proteobacteria*, *Actinobacteria*, and *Planctomycetes* were applied. Although *N. limicola* filaments are present in WWTPs their detection frequency by both methods was low with a class index of 1 in most cases. They rarely occurred at indices of 2 to 3 (Fig. 13).

The probe NLIMI 91 detecting *Trichococcus* spp. (*Firmicutes*) and the 16S rRNA sequence of an isolated ***N. limicola* I** both phylogenetically closely related to each other (Liu et al. 2000) gave positive FISH signals in 20% of the examined activated sludge samples. The detected cells were characterized by two different morphotypes, i.e. coccoid cells and short filaments. The sole long-chained *N. limicola* I identified as the dominant filamentous organism in one WWTP by classical microscopy could not be detected by FISH with the NLIMI 91 probe but showed positive signals with EUB338-mix.

The detection pattern for ***N. limicola* II** by FISH is very complex. *N. limicola* II was observed in 44% of the WWTPs by classical microscopy and gave positive FISH signals with various probes. The highest detection frequency in 30% of the WWTPs was achieved with probes **NLII65** (Fig. 14 A1 and A2) **and NLIMII 175** both detecting different 16S rRNA regions of the same organisms described as "*Candidatus* Nostocoida limicola", probably a novel genus within the A*ctinobacteria* most closely related to *Terrabacter* spp. (Blackall et al. 2000). Quantification of "*Candidatus* N. limicola" with a class index of 1 to 2 corresponded quite well with the detected morphotype *N. limicola* II. Few positive FISH signals were observed after the application of different probes (**Noli-644, PPx3-1428, and MC2-649**) targeting

various novel species ("*Candidatus* Alysiomicrobium bavaricum", "*Candidatus* Alysiosphaera europaea", "*Candidatus* Monilibacter batavus", Levantesi et al. 2004) within the class *Alphaproteobacteria* ("filamentous *Alphaproteobacteria*"). Probe **AHW183** for *N. limicola*-like organisms isolated from activated sludge, which are clustered in the *Chloroflexi* phylum (Schade et al. 2002), detected filamentous *N. limicola* II with a class index 1 and 2 in 12% and 3% of the activated sludge, respectively (Fig. 14 B1 and B2). The described morphotype *N. limicola*–like organism found in two different WWTPs with a class index of 2 could be visualized with the probes PPx3-1428 and AHW183. Furthermore it was remarkable that in some samples no *N. limicola* II morphotype was found by classical microscopy but positive FISH signals of *N. limicola* filaments were detected with the AHW183 probe and the probes specific for "filamentous *Alphaproteobacteria*" (12% of the WWTPs). In addition to these observations, none of the used specific probes, only the EUB338-mix, identified morphotype *N. limicola* II occurring with a class index of 1 in 20% of the samples analyzed.

Morphotype ***N. limicola* III** with characteristic discoid and strongly twisted filaments was detected by both classical microscopy and FISH using EUB338-mix probes but not with probe **NLIMIII 301.** This probe was designed by Liu and Seviour (2001) for a non-classical morphotype ***N. limicola* III** isolate being characterized by uniform transparent cocci in a slightly bent filament. This organism is affiliated within the *Planctomycetes* most closely related to *Isosphaera* spp. (Liu et al. 2001). FISH with NLIMIII 301 demonstrated *Isosphaera* morphotype-containing samples to exhibit probe-conferred fluorescence for single cells in the *Isosphaera* filaments only (Fig. 14 C1). Comparable FISH signals for *Isosphaera* filaments were obtained after the application of the EUB338-mix (Fig. 14 C2).

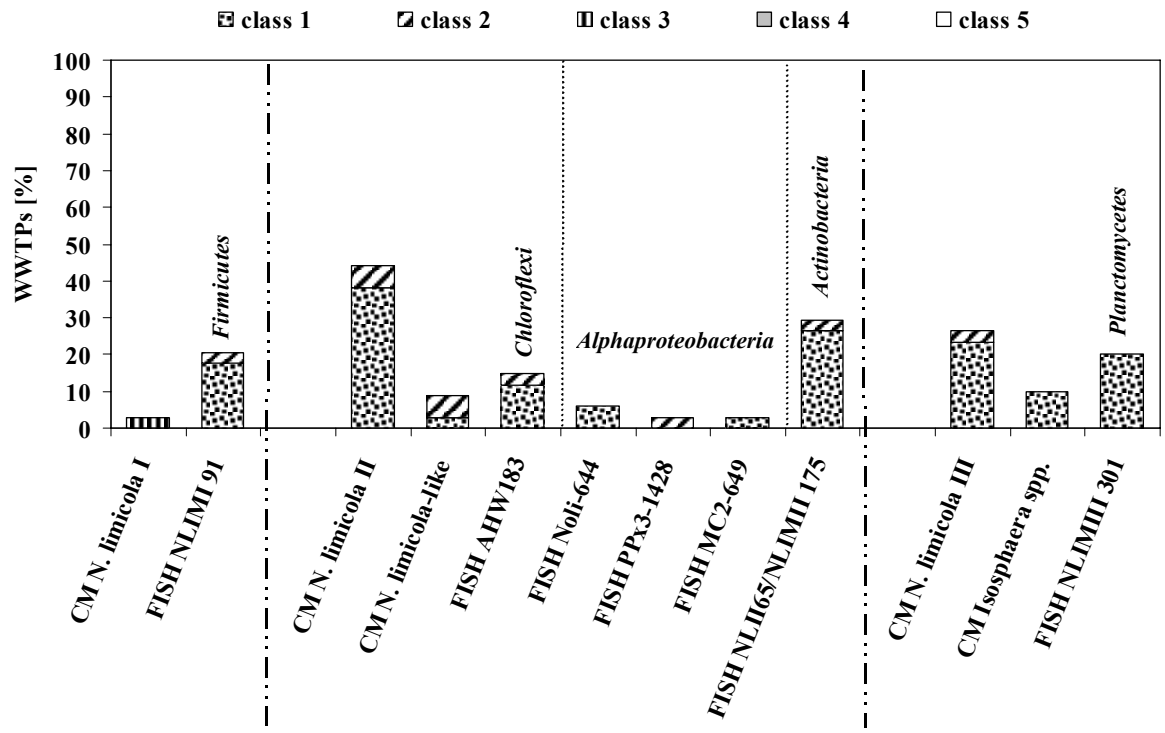


Figure 13: Detection of the morphotypes *N. limicola* I (n=34), *N. limicola* II (n=34) and *N. limicola* III (n=30) in activated sludge by classical microscopic sludge analysis (CM) and FISH (probes applied: NLIMI 91, AHW183, Noli-644, PPx3-1428, MC2-649, NLII65, NLIMII 175, NLIMIII 301).

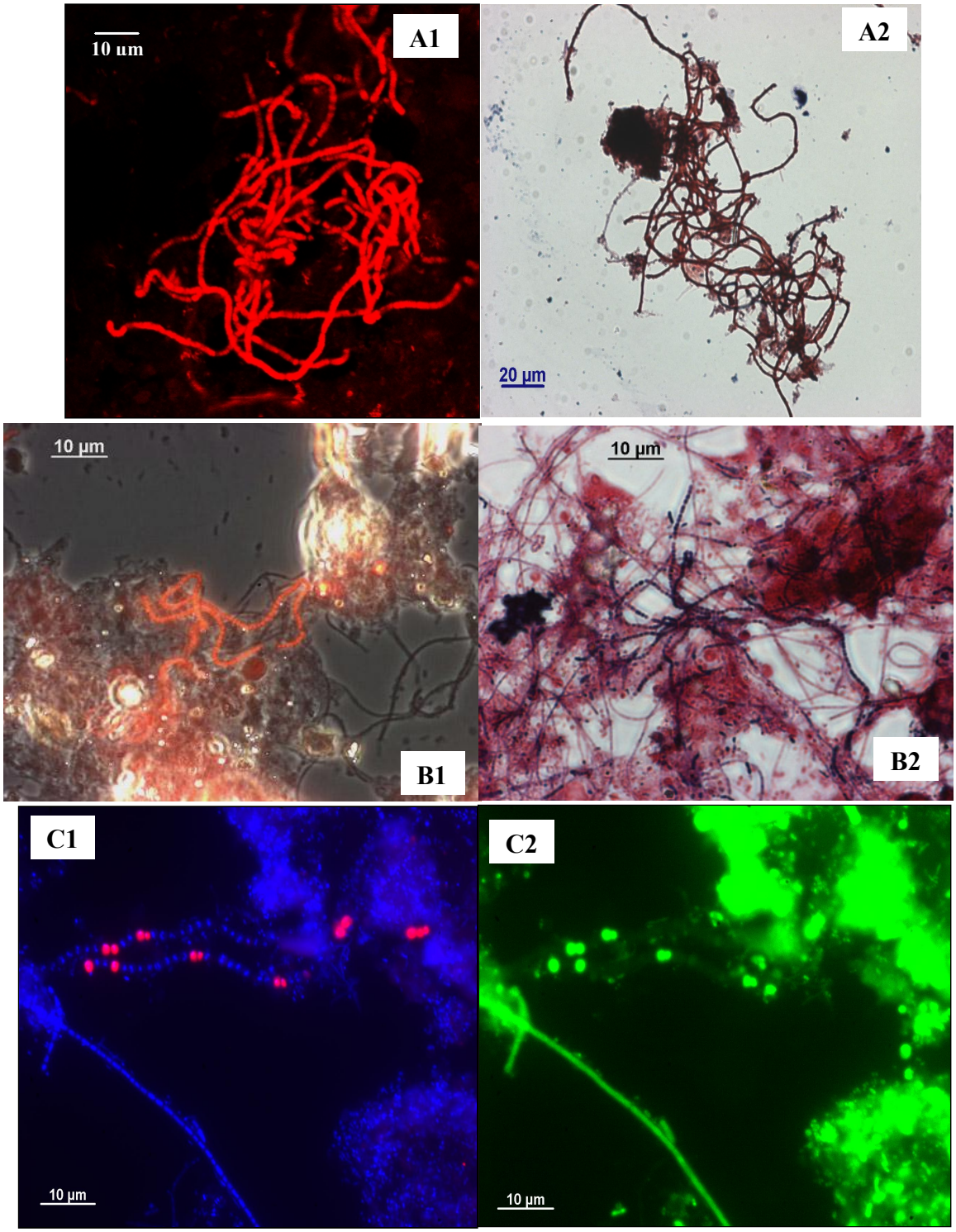
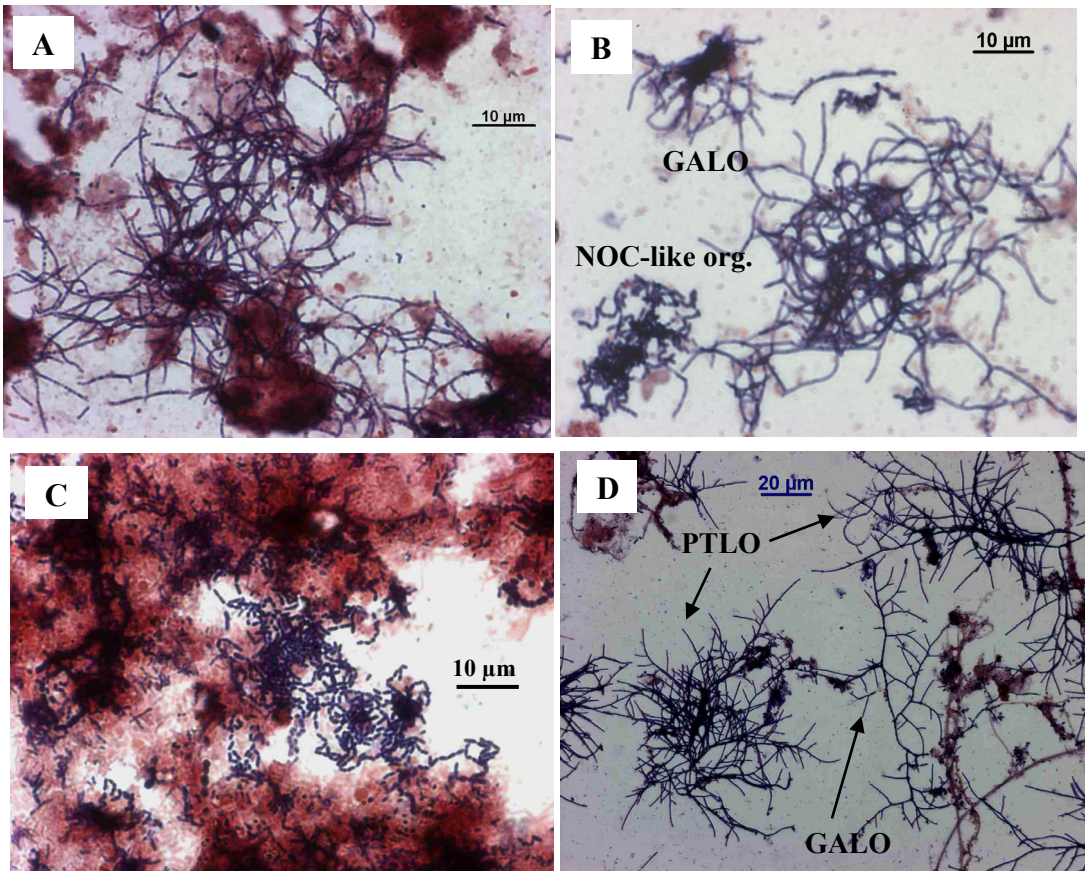


Figure 14**:** ***N. limicola*** **II** morphotype and ***Isosphaera*** **sp.** detected in activated sludge by classical methods and FISH. A CLSM image (**A1**) shows *N. limicola* II identified by CY3-labeled probe NLII65 as "*Candidatus* N. limicola" corresponding to a bright field image (**A2**) after Gram staining. An Epifluorescence image superimposed with a phase contrast image (**B1**) illustrates *N. limicola* II detected by CY3-labeled AHW183 probe (*N. limicola* isolate within the *Chloroflexi* phylum appears red). Bright field image (**B2**) shows the corresponding Gram staining (*N. limicola* II appears blue). Epifluorescence superimposed images of *Isosphaera* sp. after the application of CY3-labeled NLIMIII 301 probe (red) and DAPI staining (blue), the overlap between positive FISH and DAPI signals is magenta (**C1**) and simultaneous hybridization with FITC-labeled EUB338-mix (**C2**).

5.2 Identification of Nocardioform Actinomycetes (*Corynebacterineae*)

The screening investigations showed clearly that further research on the characterization of some scum bacteria is required. This study focused on the optimization of the identification of nocardioform actinomycetes because this bacterial group (i) appeared to be the second important scum bacteria group after *M. parvicella* and (ii) is clearly enriched in the scum layer. The main problems associated with the detection of nocardioform actinomycetes by FISH are probably limited cell wall permeability and the identification on genus and species level. Various scum samples dominated by high numbers of nocardioform actinomycetes with different morphotypes were analyzed. The scum samples were collected from two different WWTPs situated in Germany, the two-stage G-WWTP-M (AT1 and AT2) and the single-stage G-WWTP-BT (for detailed description see chapter 4.1.2). In addition, three Australian single-stage WWTPs were investigated (A-WWTP-OX, A-WWTP-TH, and A-WWTP-IN, for detailed information see chapter 4.1.2). Figure 15 shows the nocardioform actinomycetes detected after Gram staining. In Germany the branched filamentous morphotype GALO was detected in MAT2 (Fig. 15 A) and BT (Fig. 15 B). In contrast, a different morphotype, bent rods growing as non-branched filaments or in cell clusters (NOC-like organisms), was found in MAT1 (Fig. 15 C) and additionally to the GALO morphotype also in BT (Fig. 15 B). The two different branched morphologies GALO and PTLO were detected in the Australian plant TH (Fig. 15 D), and only GALO were present in the Australian plant OX (Fig. 15 E). NOC-like organisms and, in addition, Gram-positive single rods were detected in the third Australian plant IN (Fig. 15 F).



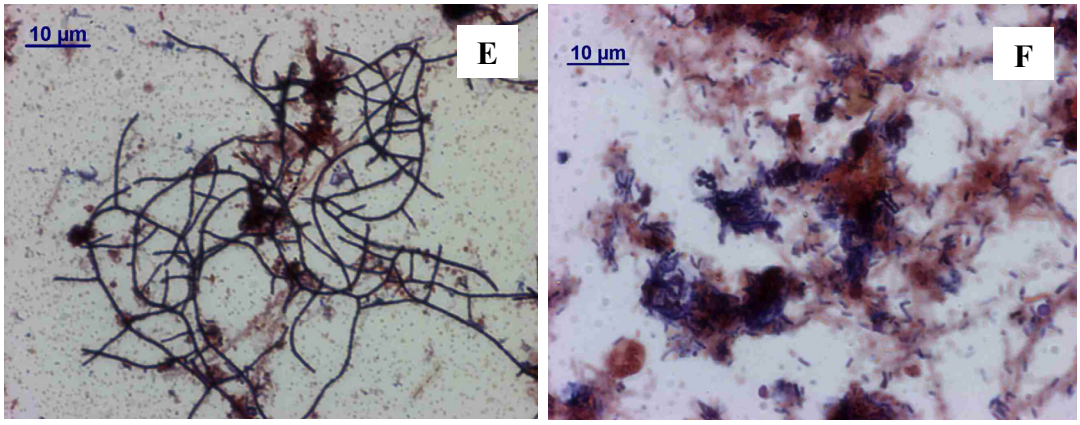


Figure 15: Bright field images of scum samples from different WWTPs after Gram staining. Nocardioform actinomycetes growing as branched filaments, **GALO**, in AT2 of G-WWTP-M (**A**) and short non-branched filaments (=**NOC-like organisms**) and **GALO** in G-WWTP-BT (**B**). **NOC-like organisms** detected in AT1 of G-WWTP-M (**C**), branched filamentous bacteria characterized by both **GALO** and **PTLO** morphotypes detected in A-WWTP-TH (**D**), **GALO** found in A-WWTP-OX (**E**), **NOC-like organisms** and **Gram-positive single rods** occurring in A-WWTP-IN (**F**).

5.2.1 Application of Different Pre-Treatment Protocols to Improve Cell Wall Permeability for FISH

The application of FISH with *Actinobacteria*- (HGC69a, HGC1156) or nocardioform actinomycetes-specific probes (MNP1 and Myc657) showed difficulties in the detection of some of the morphotypes described above. By applying lysozyme treatment recommended for Gram-positive bacteria (Beimfohr et al. 1993) and a hybridization time of 1.5 h according to Amann (1995) good FISH signals were obtained only for the NOC-like organisms found in three different WWTPs (MAT1, BT, and IN) and the GALO present in WWTP BT (Fig. 16 A-C). In contrast to these results, fluorescence signals were detected only partially for the GALO and PTLO in the two plants OX and TH, and no signals were found for the GALO of MAT2. The increase of the hybridization time to 15 h improved the FISH results for these three nocardioform actinomycete types. Detection of the whole filament was observed for GALO of the WWTPs OX and TH. Nevertheless, only a partial detection was observed for the PTLO of TH and the GALO of MAT2 after the application of the *Actinobacteria*-specific probe HGC1156.

Different permeabilization protocols described in the literature (Macnaughton et al. 1994, Davenport et al. 2000, Carr et al. 2005) were followed to optimize FISH detection for these partially visualized nocardioform actinomycetes. For all samples an increased hybridization time of 15 h was used to receive comparable results. The nocardioform actinomycetes specific-probes published were described to detect nearly the whole group but also failed to target some species (Schuppler et al. 1998, Davenport et al. 2000). For that reason the more general probe HGC1156, which was proved to target the whole group, was applied to avoid detection problems due to non-targeting probes. Only for the inconspicuous NOC-like morphotype probe MNP1 was used additionally to HGC1156 to verify that these organisms belong to the nocardioform actinomycete group. Results obtained for the different nocardioform actinomycete morphotypes exposed to the various permeabilization methods are given in Table 13.

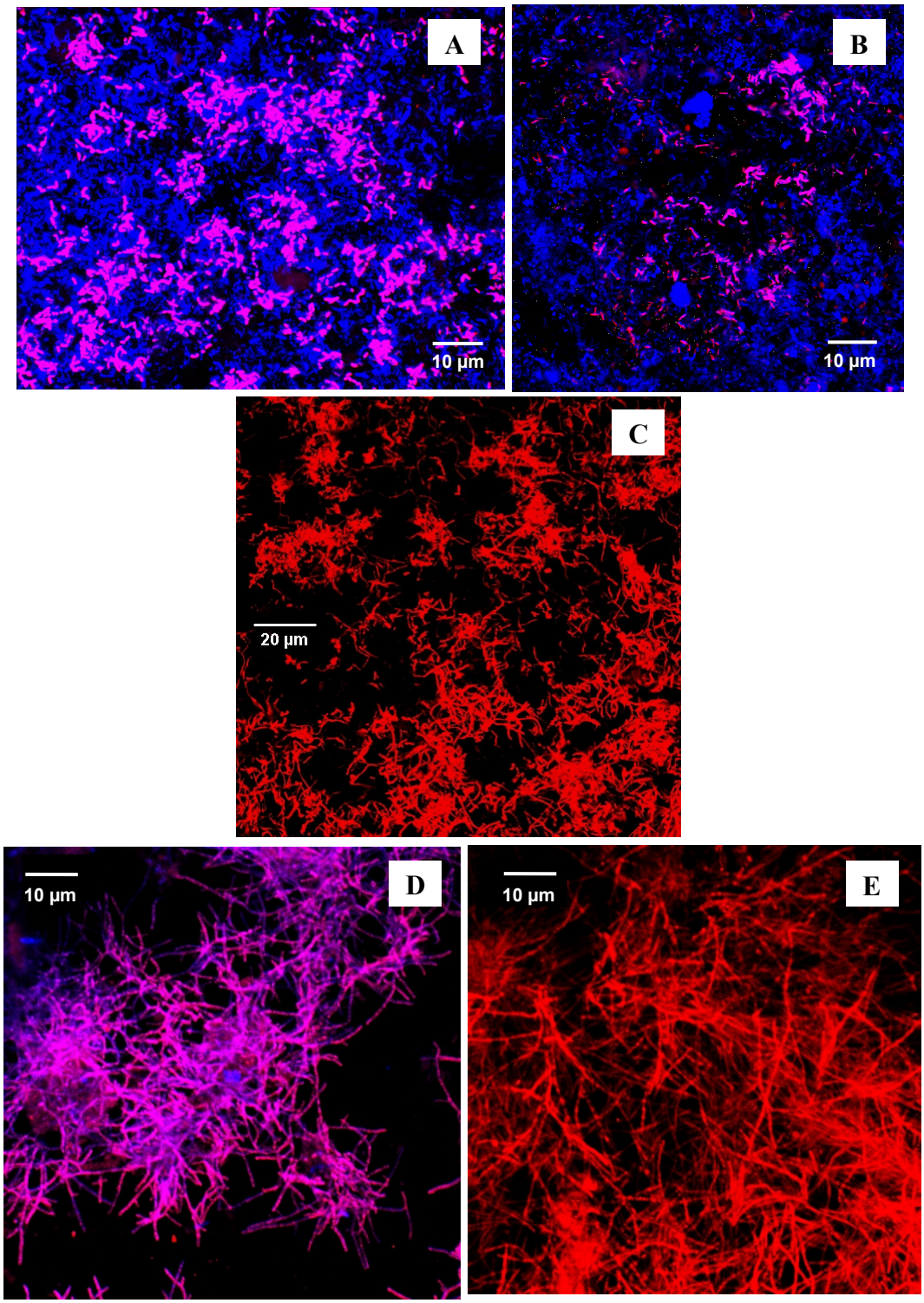


Figure 16: Nocardioform actinomycetes detected by FISH after different permeabilization protocols: **CLSM images A-C** showing **NOC-like organisms** (appear magenta) detected simultaneously by the probes MNP1-CY3 (red) and EUB338-mix-CY5 (blue), and **GALO** (appear red) identified by Myc657-CY3 (hybridization time 1.5h) after lysozyme treatment of EtOH fixed scum samples originating from different WWTPs, MAT1 (**A**), IN (**B**), and BT (**C**); **CLSM images D and E** of EtOH fixed scum samples after mild acid hydrolysis (1 M HCl) in combination with lipase/proteinase K treatment and 15 h hybridization, the morphotype **GALO** (appear magenta, **D**) in MAT2 was detected by probes HGC1156-CY3 (red) and EUB338-mix-CY3 (blue) and the morphotype PTLO (appear red, E) was visualized by HGC1156-CY3 in TH.

Table 13: FISH-signals (hybridization time 15 h) detected in different scum samples (50% EtOH fixation) enriched with nocardioform actinomycetes after different permeabilization protocols: **1** lysozyme, **2** lipase/proteinase K, **3** lysozyme/lipase/proteinase K, **4** mild acid hydrolysis (1 M HCl), **5** acid/lysozyme, **6** acid/lipase/proteinase K, **7** acid/lysozyme/lipase/proteinase K.

sample	morphotype	probe	protocol used						
			1	2	3	4	5	6	7
G-WWTP-MAT1	NOC-like org.	HGC1156	+++	+++	+++	-	-	-	-
scum 21.01.02		MNP1	+++	+++.	+++.	**n.d.**	**n.d.**	**n.d.**	**n.d.**
G-WWTP-MAT2	GALO	HGC1156	+++/-	+++/-	+++/-	+	+/-	++	++
scum 25.02.03									
G-WWTP-BT	GALO	HGC1156	+++	+++	+++	+	-	+	-
scum 13.05.03	NOC-like org.	HGC1156	+++	+++	+++	-	-	+	-
		MNP1	+++	+++.	+++	**n.d.**	**n.d.**	**n.d.**	**n.d.**
A-WWTP-OX	GALO	HGC1156	+++	-	-	-	-	-	-
scum 12.06.02									
A-WWTP-TH	GALO	HGC1156	+++	+++/-	++/-	-	-	?	?
scum 12.06.02	PTLO	HGC1156	+++/-	+++/-	++/-	++	+	++	++
A-WWTP-IN	NOC-like org.	HGC1156	+++	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**
scum 12.06.02		MNP1	+++	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**

+++ strong fluorescence signal, whole filament
++ moderate fluorescence signal, whole filament
\+ weak fluorescence signal, whole filament
+++/- partially strong fluorescence signal
\- no fluorescence signal
n.d. not determined

The NOC-like organisms gave strong fluorescence signals after different enzymatic treatments, e.g. lysozyme, the combination of lipase and proteinase K, or the application of all three enzymes, as was shown for the scum samples originating from the two German WWTPs (MAT1 and BT). Mild acid hydrolysis alone and the combination of hydrochloric acid with different enzymes resulted in NOC-like organisms to show very weak or no FISH signals at all. Nocardioform actinomycetes developing branched filaments characterized as GALO or PTLO responded differently to the various permeabilization methods. GALO in the scum samples from BT responded to the various pre-treatments similarly as did NOC-like organisms with optimal FISH signals after lysozyme treatment or the combination of different enzymes. In contrast, for the GALO found in the two Australian WWTPs OX and TH the most effective permeabilization protocol was lysozyme treatment alone. Strong but only partial fluorescence signals were achieved for the GALO from MAT2 and the PTLO from TH after enzymatic pre-treatment as was mentioned above. Mild acid hydrolysis alone or in combination with lipase/proteinase K effected the detection of the whole filaments but the signal intensity was distinctly lower (Fig. 16 D and E). Further it was observed after applying probes specific for the domain *Bacteria* hydrochloric acid treatment cause cell lysis of most bacteria. This makes the exact quantification against the total bacteria populations impossible and only an estimation of the occurrence of these nocardioform actinomycetes can be carried out.

5.2.2 Identification of Nocardioform Actinomycetes by FISH using Specific Probes

The nocardioform actinomycete group is characterized by a high taxonomic diversity. Hence it is of interest to know whether the morphotypes found constitute the same or various organisms. The observed varying response to several permeabilization protocols might indicate that different genera are involved. However, it might also mean that different environmental conditions influence cell wall permeability. Different available probes specific for *Actinobacteria* (HGC69a, HGC235) to get a general overview, specific for the group nocardioform actinomycetes (MNP1, Myc657), specific for the genus *Gordonia* (Gor596) and specific for *Skermania piniformis* (NPI425) were applied to get more detailed information. For all samples the lysozyme pre-treatment (protocol P1) and the hybridization time of 15 h was used because these treatments resulted in the strongest FISH signals. Additionally the permeabilization protocol including hydrochloric acid in combination with lipase/proteinase K (protocol P6) was used for the samples being enriched with nocardioform actinomycetes that were partially detected after lysozyme treatment.

All nocardioform actinomycetes found in different scum samples were detectable with the various *Actinobacteria* probes. The fluorescence signal intensity for the HGC235 was distinctly lower as compared to the other probes despite the application of the helper probe HGC270H, which is recommended to increase the FISH signals (Fuchs et al. 2000). The NOC-like organisms in all three samples were very well identified by the probes MNP1 and Myc657. GALO present in BT and the two Australian plants OX and TH were also detected by both probes MNP1 and Myc657. None of the more specific probes such as Gor596 and NPI425 were able to detect GALO from BT. In contrast, GALO originating from the Australian plants were identified as *Gordonia* spp. (Fig. 17 A and B). The investigation of GALO growing in MAT2 and the PTLO found in TH demonstrated different results. Probe MNP1 failed to target these organisms. The PTLO were detected by the group-specific probe

Myc657 and probe NPI425 specific for *Skermania piniformis*. For both probes, as expected, only partially FISH signals of the filaments were observed after lysozyme pre-treatment. However, also after the permeabilization protocol P6 (1 M HCl and lipase/proteinase K), which allowed detection of whole filaments using probe HGC1156, only fragments of the PTLO filaments gave positive FISH signals with NPI425 (Fig. 17 C). Simultaneously, the whole filaments were detected by probes for the domain *Bacteria*. In addition to the *Actinobacteria*-specific probes, only probe Myc657 was able to detect GALO present in the scum samples from MAT2. However, the detection by Myc657 was not very clear because after the lysozyme pre-treatment only a very low fraction of the filaments could be detected and very weak FISH signals were obtained using mild acid hydrolysis in combination with lipase/proteinase K.

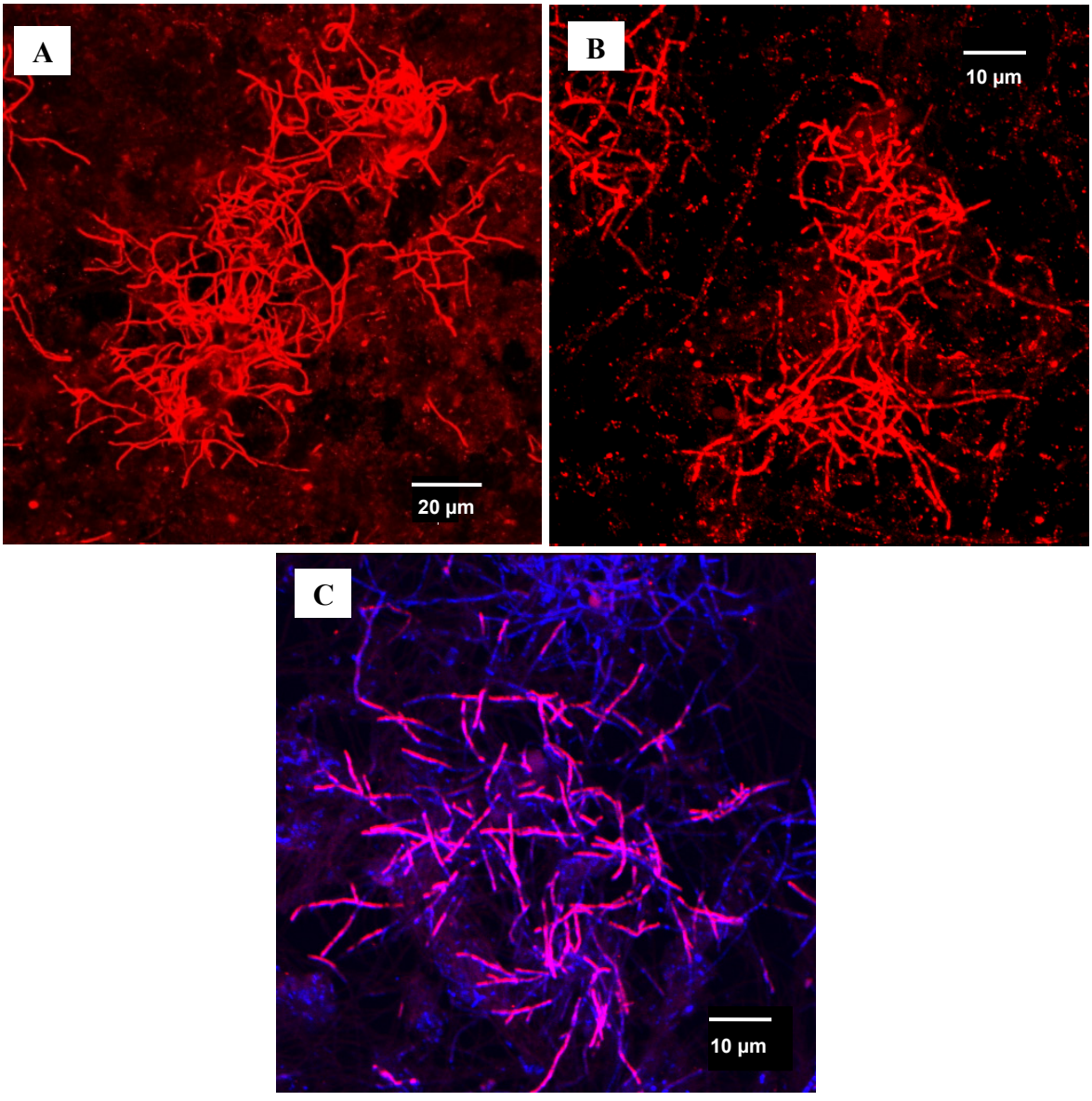


Figure 17: FISH with specific probes detecting different species within the nocardioform actinomycete group. **CLSM images: *Gordonia* spp**. found in both Australian plants, OX **(A)** and TH **(B),** gave positive signals after lysozyme treatment of EtOH fixed scum samples and the application of CY3-labeled probe Gor596. ***Skermania piniformis*** growing in the scum samples from TH detected by the CY3-labeled probe NPI425 (red) and simultaneously hybridized with CY5-labeled probes EUB338-mix (blue), after mild hydrochloric acid treatment in combination with lipase/proteinase K treatment of EtOH fixed scum samples. The overlap between the FISH signals of NPI425 and EUB338-mix probes are shown as magenta **(C).**

5.2.3 Full-cycle 16S rRNA Approach and DGGE to Identify Nocardioform Actinomycetes at Genus and Species Level

The identification of nocardioform actinomycetes by applying specific probes was only partially successful. The results indicated that further molecular biological techniques are required to obtain more phylogenetic knowledge. For that reason the full-cycle 16S rRNA approach was carried out for the scum samples originating from A-WWTP-TH dominated by PTLO that were not clearly identified as *Skermania piniformis*, and for all scum samples collected from the German WWTPs that were enriched with different GALO and NOC-like organisms. For both morphotypes no information at genus and species level could be received after the application of published probes as was shown above. Additionally DGGE was used to get further information about the diversity of the nocardioform actinomycetes within the different scum samples.

5.2.3.1 DNA extraction and clone libraries

The DNA extraction method was chosen very carefully due to the very complex cell wall of the nocardioform actinomycetes. It was shown that a very high amount of DNA was obtained for all scum samples after applying the specific extraction procedure of the FASTDNA SPIN Kit BIO101 (for detailed description see chapter 4.2.5). The positive PCR amplification with the *Actinobacteria*-specific primers Act235f and AB1165r proved that the extracted DNA included *Actinobacteria* species. Extracted DNA from the four different scum samples was used as a mixed template for PCR-amplification of approximately the whole 16S rRNA gene with universal 16S rRNA primers (27f and 1492r). Cloning of the PCR products of each sample resulted in four different 16S rDNA clone libraries. The clone libraries were named TH, MAT1, MAT2, and BT, detailed information is given in Table 14.

Table 14: Different clone libraries and their corresponding origin of scum samples characterized by various nocardioform actinomycete (NOC) morphotypes.

name of the clone library	origin of the DNA (scum samples)	sampling date	NOC morphotype
TH	Australian WWTP-TH	12.06.02	PTLO, GALO
MAT1	German WWTP-MAT1	22.01.02	NOC-like org.
MAT2	German WWTP-MAT2	25.02.03	GALO
BT	German WWTP-BT	13.05.03	GALO, NOC-like org.

5.2.3.2 Screening and RFLP profiling of the clone libraries

A total of 65 clones containing the correct insert size from the clone library TH were examined by RFLP. They were found to belong to 35 different RFLP groups. Twenty-one RFLP groups were each composed of individual clones. The remaining 14 RFLP groups corresponded mostly to 2 up to 5 clones. These results indicate that a high taxonomic diversity might be present in the clone library TH.

Because of the high numbers of RFLP groups found in the clone library TH and the focus on *Actinobacteria* in this study, the clone libraries MAT1, MAT2, and BT were screened with *Actinobacteria*-specific primers before RFLP profiling. The selection of an *Actinobacteria*-specific primer pair was performed with regard to the availability of corresponding FISH probes confirming that the organisms of interest (different morphotypes of nocardioform actinomycetes) possess the specific target sequence. Therefore for the screening study of the clone libraries the specific primers Act235f (Stach et al. 2003) and AB1165r (Lüdemann and Conrad 2000) were used. These primers target similar 16S rRNA regions as the *Actinobacteria*-specific probes HGC235 and HGC1156 (Erhart 1997), which visualized the nocardioform actinomycetes of interest by FISH. From a total of 100 clones for each clone library MAT1 and MAT2 a positive PCR reaction with *Actinobacteria*-specific primers was observed for 54 and 44 clones, respectively. Within the clone library BT from 50 clones analyzed by the *Actinobacteria*-specific primers 37 clones produced positive PCR amplicons.

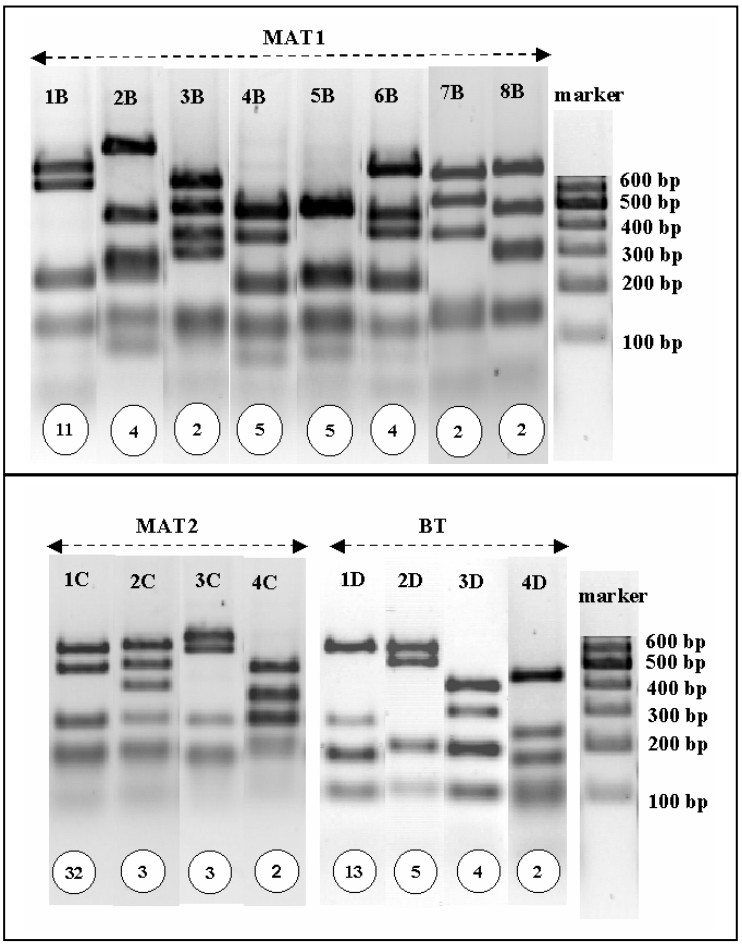


Figure 18: Profiles of the different RFLP groups composed of at least two or more clones of the clone libraries MAT1, MAT2, and BT. RFLP groups are named 1B–8B (MAT1), 1C-4C (MAT2), and 1D-4D (BT) as is indicated above the profiles. Numbers of corresponding clones are indicated below each profile.

Twenty-six, 9, and 16 different RFLP groups were detected for *Actinobacteria*-positive clones from the various clone libraries MAT1, MAT2, and BT, respectively. Distinctly lower numbers of 8, 4, and 4 RFLP groups corresponding to at least two or more clones were found for MAT1, MAT2, and BT, respectively (Fig. 18). The dominant RFLP groups were composed of 11 (MAT1), 32 (MAT2), and 13 (BT) clones corresponding to 20%, 73%, and 35% of the analyzed clones, respectively. The comparison of the profiles from the different clone libraries demonstrated a similar RFLP group within clone libraries MAT1 and BT, i.e. group 1B and 2D, indicating the same organisms to be present in both libraries.

5.2.3.3 Phylogenetic sequence analysis I – partial sequences of clone inserts

Clone inserts of representative clones from all RFLP groups composed of several clones for all four clone libraries were partially sequenced. In addition, clone inserts of some clones corresponding to individual RFLP groups for each clone library were analyzed.

Table 15: Phyla of the domain *Bacteria* represented in the clone library TH determined by BLAST (Basic Local Alignment Search Tool, Altschul et al. 1990) comparison of partial clone insert sequences (36 clones, 530f primer).

phylum	% of clone library
Proteobacteria	**39**
Alphaproteobacteria	6
Betaproteobacteria	28
Gammaproteobacteria	3
Epsilonproteobacteria	3
Bacteroidetes	**31**
Flavobacteria	11
Sphingobacteria	14
unclassified *Bacteroidetes*	6
Planctomycetes	**6**
Actinobacteria	**22**
Corynebacterineae	8
Propionibacterineae	8
unclassified *Actinobacteria*	6
Acidobacteria	**3**

In total 36 clones were sequenced within the clone library TH. The BLAST evaluation of these partial sequences demonstrated that clones within the same RFLP group were often affiliated with different taxonomic groups. The groups that the analyzed clones were accommodated are shown in Table 15. The majority of the clone sequences grouped within the *Proteobacteria* phylum (39%), followed by the *Bacteroidetes* phylum (31%). The *Actinobacteria* phylum was the third frequent group represented by 22% of the analyzed clones. These clones were affiliated with the *Corynebacterineae* (nocardioform actinomycetes), *Propionibacterineae*, and unclassified *Actinobacteria*. The three clones belonging to the *Corynebacterineae* were all classified as *Skermania piniformis* with 98 to 99% similarity. The closest published relatives of the other *Actinobacteria* clones were *Microlunatus phosphovorus* (90 to 94% similarity) and "*Candidatus* M. parvicella" (90%

similarity). Within the clone library TH no *Gordonia*-related sequence was detected despite the positive FISH result received after the application of *Gordonia*-specific probe Gor596 (see chapter 5.2.2).

Results from BLAST comparison for all partial sequences of the clone libraries MAT1, MAT2, and BT are given in Table 16. The results are sorted according to RLFP groups and information about the phylogenetic classification and the closest published relatives is listed. In total, 21, 12, and 11 clones of the clone libraries MAT1, MAT2, and BT, respectively, were analyzed. Despite screening with *Actinobacteria*-specific primers some clones belong to other taxonomic phyla, e.g. *Proteobacteria* and *Bacteroidetes*. Approximately 80%, 75%, and 67% of the sequenced clones within the clone libraries MAT1, MAT2, and BT were affiliated with the *Actinobacteria* phylum, respectively. Specificity might be improved by a more stringent PCR reaction with a higher annealing temperature of more than 58°C. However, it was demonstrated on pure culture studies that PCR conditions using a higher annealing temperature limited the detection of some members of the *Actinobacteria* phylum (data not shown).

Twenty-nine percent of the sequenced clones within the clone library MAT1 were classified as *Corynebacterineae* (nocardioform actinomycetes) and all these clones fell into the dominant RFLP group (1B) composed of 11 clones. Four of these clone sequences closely related to each other (99.8 to 100% similarity) matched sequences from uncultured bacterium clones found in scum samples (Wagner and Cloete 2002) with 99% similarity and showed 98% similarity to *Dietzia maris*. Two clone sequences of the RFLP group 1B were affiliated with a different genus both being closely related to *Gordonia hirsuta* and *Gordonia amarae* with the same similarity of 97%. Other *Actinobacteria* clones of interest might be clones hat are closely related to "*Candidatus* N. limicola" and "*Candidatus* M. parvicella". These organisms were detected in scum samples as was shown in the screening investigations.

The majority of the sequences within clone library MAT2 were classified within the *Corynebacterineae* group (58%). The three analyzed clones of the dominant RFLP group 1C including a total of 32 clones were closely related to three different *Rhodococcus* spp. isolated from foaming sludge (Soddell et al. 1998) with a similarity of 99%. Other *Corynebacterineae* clones corresponding to different RFLP groups (2C and 3C) are closely related to *Nocardia corynebacterioides* (97%) and *Gordonia spumae* (99%), respectively. Two clones (RFLP group 4C and 7C) affiliated with the *Actinobacteria* but not with the *Corynebacterineae* showed a 90% sequence similarity to "*Candidatus* M. parvicella" and a 98% sequence similarity to *Microbacterium* sp.

Table 16: Phylogenetic classification of the partial sequences originating from the clone libraries MAT1, MAT2, and BT by BLAST; (BLAST = Basic Local Alignment Search Tool, Altschul et al. 1990).

clone library	RFLP group (no. of profiles)	clone name	phylogenetic classification	closest published relatives (accession number)	similarity [%]
MAT1	1B (11)	AT1-110, AT1-127, AT1-132, AT1-228	*Actinobacteria, Corynebacterineae*	uncultered bacterium clone, 10, 53 (AF513105, AF513099)	99
				Dietzia maris (AY167849)	98
		AT1-111, AT1-315		*Gordonia hirsuta* (X93485)	97
				Gordonia amarae (AF020329)	97
MAT1	2B (4)	AT1-113, AT1-325	*Gammaproteobacteria*	uncultured gammaproteobacterium clone (AB074615)	98
				Lysobacter gummosus (AB166894)	97
MAT1	3B (2)	AT1-121, AT1-217	*Actinobacteria, Propionibacterineae*	*Propioniferax innocua* (AF227165)	96
MAT1	4B (5)	AT1-130	*Actinobacteria*	uncultured actinobacterium clone (AY500111)	94
				"*Candidatu* M. parvicella" (X93044)	91
		AT1-220, AT1-312	*Actinobacteria*	uncultured bacterium (AJ504573)	98
			Micrococcineae, Intrasporangiaceae	"*Candidatus* N. limicola" (Y14597, X85212)	97
MAT1	5B (5)	AT1-134	*Actinobacteria, Bifidobacteriaceae*	*Bifidobacterium bifidum* (S83624)	99
		AT1-232	*Actinobacteria, Propionibacterineae*	*Nocardioides* sp. (AJ244655)	97
MAT1	6B (4)	AT1-139, AT1-149, AT1-29, AT1-249	*Actinobacteria*	uncultured actinobacterium clone (AY500111)	94
				"*Candidatus* M. parvicella" (X93044)	91
MAT1	7B (2)	AT1-330	*Betaproteobacteria*	*Acidovorax* sp. (AF235010)	98
MAT1	8B (2)	AT1-320	*Actinobacteria, Propionibacterineae*	*Tessaracoccus bendigoensis* (AF038504)	97
MAT2	1C (32)	AT2-130, AT2-25, AT2-253	*Actinobacteria, Corynebacterineae*	*Rhodococcus* sp. (X85241, X85242. X852409	99
MAT2	2C (2)	AT2-15, AT2-242	*Actinobacteria, Corynebacterineae*	*Nocardia corynebacterioides* (AY167850)	97
MAT2	3C (3)	AT2-119, AT2-218	*Actinobacteria, Corynebacterineae*	*Gordonia spumae* (AJ251724)	98

Table 16 continued.

clone library	RFLP group (No. of profiles)	clone name	phylogenetic classification	closest published relatives (accession number)	similarity [%]
MAT2	4C (1)	AT2-127	*Actinobacteria*	uncultured actinobacterium clone (AY500111) "*Candidatus* M. parvicella" (X93044)	96 90
MAT2	5C (1)	AT2-124	*Alphaproteobacteria*	uncultured alphaproteobacterium clone (AY139005) *Stella vacuolata* (AJ535711)	97 91
MAT2	6C (1)	AT2-148	*Bacteroidetes*	*Pedobacter saltans* (AJ438173)	91
MAT2	7C (1)	AT2-113	*Actinobacteria, Micrococcineae*	*Microbacterium* sp. (AF306542)	98
MAT2	8C (1)	AT2-239	*Bacteroidetes*	uncultured *Bacteroidetes* bacterium (AY038763) *Sphingobacterium* sp. (AY336117)	95 93
BT	1D (13)	BT-12, BT-118	*Actinobacteria, Corynebacterineae*	*Rhodococcus erythropolis* (AY281114)	100
BT	2D (5)	BT-127	*Actinobacteria, Corynebacterineae*	uncultered bacterium clone, 10, 53 (AF513105, AF513099) *Dietzia maris* (AY167849)	100 98
BT	3D (2)	BT-120	*Actinobacteria*	"*Candidatus* M. parvicella" (X93044)	99
BT	4D (2)	BT-117 BT-145	*Actinobacteria, Corynebacterineae*	*Mycobacterium* sp. (AY147261)	98
BT	5D (1)	BT-22	*Actinobacteria, Corynebacterineae*	*Dietzia sp*. (YO8313)	98
BT	6D (1)	BT-19	*Actinobacteria, Corynebacterineae*	*Dietzia maris* (AY167849) *Rhodococcus erythropolis* (AY281114)	97 96
BT	7D (1)	BT-130	*Bacteroidetes*	*Sphingobacterium* sp. (AY167837)	94
BT	8D (1)	BT-128	*Betaproteobacteria*	*Aquaspirillum metamorphum* (Y18618)	98

Within the clone library BT 58% of the sequenced clones belonged to the *Corynebacterineae*. Two clones of the dominant RFLP group 1D (composed of 13 clones) were sequenced and showed a 100% sequence similarity to *Rhodococcus erythropolis*. The one sequenced clone of the RFLP group 2D was affiliated within the *Corynebacterineae* and matched uncultured bacterium clones detected by Wagner and Cloete (2002) with a 100% similarity, and was related by 98% to *Dietzia maris*. This clone sequence (BT-127) showed a 100% similarity to the clone sequence MAT1-132 within the dominant RFLP group 1B of the clone library MAT1. These results confirmed the suggestion that both RFLP groups, 1B and 2D, contained the same organism because of similar profile patterns. Furthermore two other clones within the clone library BT, corresponding to different RFLP groups (5D and 6D), showed a 100 % (BT-22) and 95% (BT-19) similarity to clone BT-127. They were both closely related to *Dietzia maris*. However, clone BT-19 was closely related to two different species, *Dietzia maris* and *Rhodococcus erythropolis*. The CHIMERA CHECK analysis within the Ribosomal Database Project (Cole et al. 2003) of clone BT-19 showed that this sequence was chimeric, consisting of two different 16S rDNA fragments (fragment 1 = first 540 bases showed 94% similarity to *Rhodococcus erythropolis* and fragment 2 = bases from 541 to 744 matched 100% with the sequence of *Dietzia maris*). Further sequences of interest within the clone library BT, corresponding to the RFLP groups 3D and 4D, were closely related to "*Candidatus* M. parvicella" (99%) and *Mycobacterium mageritense* (98%), respectively.

BLAST results of the partial sequences of all clone libraries demonstrated that the RFLP profiling of clone libraries MAT1, MAT2, and BT was quite successful. Furthermore the majority of the *Actinobacteria*-positive clones belonged to the *Corynebacterineae* with high similarity to *Dietzia* spp. and different *Rhodococcus* spp.. A high diversity was detected within the clone library TH and only clones closely related to *Skermania piniformis* were grouped within the *Corynebacterineae*.

5.2.3.4 Profiling of the clone libraries and environmental DNA by DGGE

Simultaneous DGGE analysis of PCR products from clone inserts and those obtained from environmental DNA might give an indication of the representative members in the natural microbial community. This approach was carried out for the clone libraries containing clone inserts belonging to several members within the *Corynebacterineae* group, which was determined for the clone libraries MAT1, MAT2, and BT. All clones with clone inserts affiliated within the *Corynebacterineae* group and clones that were closely related to scum bacteria such as "*Candidatus* M. parvicella"and "*Candidatus* N. limicola" were analyzed. In this study DNA fragments smaller than 200 bp were used for the DGGE approach because experience showed longer fragments to cause problems with band separation (data not shown). Hence the clone inserts were reamplified with the nested primers 342f and 518r. Environmental DNA of each clone library was first amplified with the *Actinobacteria*-specific primers Act235f and AB1165r. These PCR amplicons were reamplified with the nested primers 342f and 518r.

Different specific DGGE patterns within the *Actinobacteria* group were produced from the three environmental DNA types (originating from different scum samples MAT1, MAT2, and BT, Fig. 19). But there are also some similarities, e.g. the main band of the MAT1-DNA showed also up within the BT-DNA pattern.

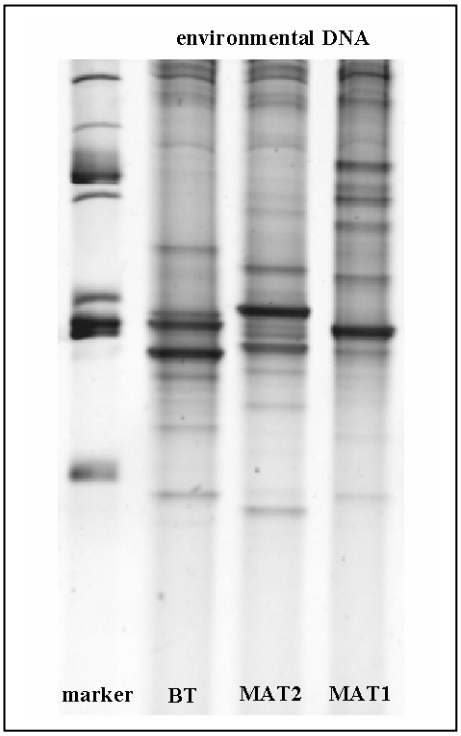
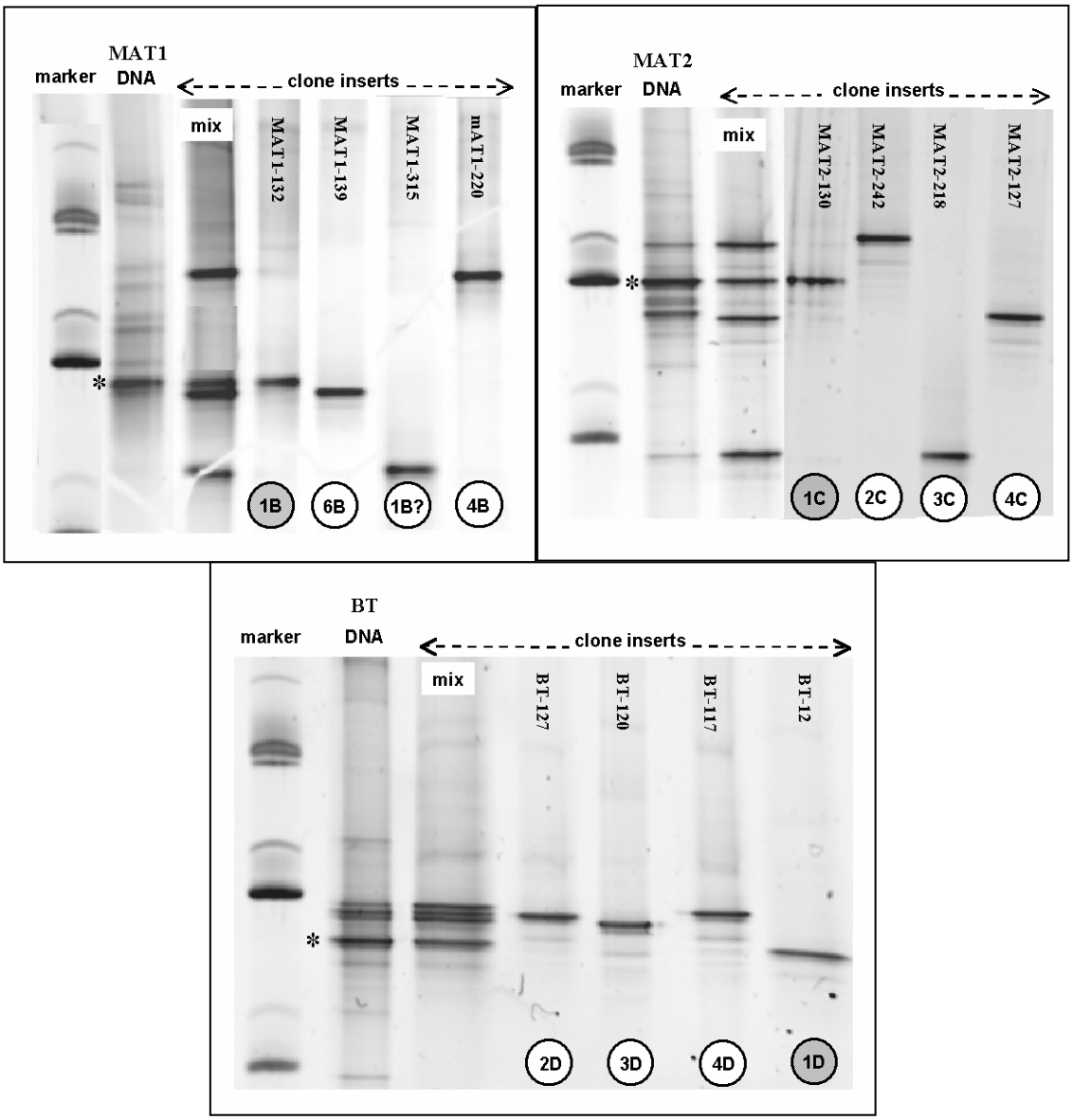


Figure 19: DGGE patterns of 16S rRNA gene sequences (PCR with *Actinobacteria*-specific primers Act235f and AB1165r, and nested primers 342f and 518r) obtained from environmental DNA originating from scum samples of different WWTPs (MAT1, MAT2, and BT). Gel gradient ranging from 50 to 70% denaturant.

The comparison of the DGGE results of the clone inserts with respective environmental DNA is shown in Figure 20. The four chosen clone inserts of each clone library yielded single strong bands. Individual clones were clearly reflected in the DNA patterns of the respective clone mixtures. The clone mixture-specific patterns of the clone libraries MAT2 and BT revealed similar patterns comparable to the corresponding environmental DNA. However, for both environmental DNAs one main DNA band was detected that could be correlated to the dominant clones MAT2-130 (closely related to three different *Rhodococcus* spp.) and BT-12 (classified as *R. erythropolis*), respectively. Only one strong band signal was visualized in the environmental DNA originating from the WWTP-MAT1. This band corresponded with the dominant clone MAT1-132 (closely related to *Dietzia maris*). Two weak bands within the environmental DNA-specific pattern of WWTP-MAT1 might represent the clone insert of MAT1-220 (next published relative "*Candidatus* N. limicola") and MAT1-139 (next published relative "*Candidatus* M. parvicella"). No corresponding band within the environmental DNA pattern was found for the clone insert MAT1-315 (closely related to *Gordonia* spp.).

Figure 20: DGGE profiles of single clone inserts (using nested primers 342f and 518r) and of clone mixtures of the different clone libraries MAT1, MAT2, and BT. Additionally the DGGE patterns of the respective environmental DNA from each clone library are shown. Clone names are written above each individual lane and the corresponding RFLP groups are listed below. The dominant RFLP groups of each clone library are labeled in gray. The bands marked with a star were excised and sequenced. Gel gradient ranging from 50 to 70% denaturant.



5.2.3.5 Phylogenetic sequence analysis II - sequences of DGGE bands of environmental DNA and nearly full-length sequences of clone inserts

The DGGE results indicate that clone inserts of the dominant clones within each clone library MAT1, MAT2, and BT might represent the major *Actinobacteria* organisms within the respective habitat. However, it must be stated that bands at the same position in the gel have the same melting behavior but not necessarily the same sequence. Therefore the main DGGE bands (see Fig. 20, labeled with a star) of the environmental DNA patterns, which correspond to the specific DNA bands of the dominant clone inserts within each clone library, were excised and sequenced. Direct pairwise sequence comparisons between the sequences of the main environmental DNA-DGGE bands and the corresponding clone inserts are shown in Table 17. The sequence similarity between the dominant clones within the clone libraries MAT2 and BT, and the respective environmental DNA-DGGE band was 100%, while those between the dominant clone within the clone library MAT1 and the main DGGE band of the respective environmental DNA was 96%. These results demonstrate that the sequence inserts

of the dominant clones within the clone libraries MAT2 and BT represent the major *Actinobacteria* species in the scum samples. In contrast, different species of the same genus to which the dominant clones within the clone library MAT1 belong might be found in the scum samples from MAT1.

Table 17: Similarity matrix (RDP II, Cole et al. 2003) showing the % similarity among 16S rDNA sequences of the main DGGE bands from the environmental DNA (MAT1, MAT2, and BT), the dominant clones for the corresponding clone library, and the closest relatives.

MAT1

strain no.	DGGE band / clone / species (accession no.)	% sequence similarity with species of strain no.		
		1	2	3
1	main DGGE band (DNA-MAT1, 140 bp)			
2	MAT1-132	**96.0**		
3	*Dietzia maris* (AY167849)	**96.0**	**98.5**	
4	*Dietzia* sp. (AF481211)	**95.2**	**98.5**	**98.7**

MAT2

strain no.	DGGE band / clone / species (accession no.)	% sequence similarity with species of strain no.		
		1	2	3
1	main DGGE band (DNA-MAT2, 140 bp)			
2	MAT2-130	**100**		
3	*Rhodococcus* sp. (X85242)	**100**	**99.9**	
4	*Rhodococcus* sp. (X85241)	**100**	**99.9**	**100**

BT

strain no.	DGGE band / clone / species (accession no.)	% sequence similarity with species of strain no.	
		1	2
1	main DGGE band (DNA-BT, 140 bp)		
2	BT-12	**100**	
3	*Rhodococcus. erythropolis* (AY281114)	**100**	**100**

Near complete insert sequences were determined for two clones within the clone library TH (TH-133 and TH-146) that were closely related to the scum bacteria *Skermania piniformis* and "*Candidatus* M. parvicella". Within the clone libraries MAT1, MAT2, and BT the dominant clones (MAT1-132, MAT2-130, and BT-12) were analyzed for full-length sequences. In addition, full-length sequences were obtained for the clones corresponding to distinct DGGE bands within the pattern of each environmental DNA (MAT2-242, MAT2-218, MAT2-127, BT-127, BT-120, and BT-117). None of the full-length sequences was determined as potential

chimera (CHIMERA CHECK, RDP II, Cole et al. 2003). Therefore all sequences were phylogenetically analyzed (tree reconstruction and sequence similarity analysis) using the ARB software package and database (Ludwig et al. 2004). The phylogenetic relationships within the reconstructed phylogenetic tree (Fig. 21) supported the crude classification of the partial sequences using BLAST (see Table 16). For the three clones BT-120, TH-146, and MAT2-127 the closest named published relative represents "*Candidatus* M. parvicella". While clone BT-120 clusters within the *Microthrix* group (98% sequence similarity), clones TH-146 and MAT2-127 belong to distinctive phylogenetic groups. The low sequence similarity of these clones to "*Candidatus* M. parvicella" (90%) and to other published organisms, e.g. *Acidimicrobium ferrooxidans* (88%), confirmed that these organisms might belong to two new taxonomic clades. All three sequences were targeted with *Actinobacteria*-specific probes HGC1156 and HGC235 but only the BT-120 sequence had no mismatches with the *M. parvicella*-specific probe MPA645.

All other clone sequences were placed within the *Corynebacterineae* suborder (nocardioform actinomycetes) and grouped into five genera, *Dietzia*, *Gordonia, Mycobacterium, Rhodococcus*, and *Skermania*. The tree topology demonstrates that these genera forming individual coherent clades and the species of each clade exhibit 16S rDNA homology values of more than 94.9%. *Rhodococcus* represents a relatively divergent genus. The study of Rainey et al. (1995a) showed the *Rhodococcus* species to fall into six distinct groups. Three different *Rhodococcus* spp. were found within the clone libraries MAT2 and BT. Clone BT-12 (dominant clone) fell into the *Rhodococcus erythropolis* group with a sequence similarity of 99.5% to *R. erythropolis*. The *Rhodococcus*-related clones (MAT2-130 and MAT2-242) within the clone library MAT2 were not affiliated to any of Rainey´s *Rhodococcus* groups. Both clones clustered in independent lines of descent within the radiation of *Rhodococcus* with a sequence similarity to each other of 96.7%. The dominating clone (MAT2-130, 32% of the clones analyzed) grouped together with *Rhodococcus* species isolated from foaming activated sludge by Soddell et al. (1998) with a sequence similarity of > 99.7% (*Rhodococcus* cluster A). In contrast, the second clone (MAT2-242) represented only 6% of the analyzed clones but produced a distinct band in the DGGE profile forming a single branch (*Rhodococcus* cluster B). The closest published relative to clone MAT-242 was *Nocardia corynebacterioides* (synonym *Rhodococcus corynebacterioides)* with a sequence similarity of 97%. The sequences of both *Rhodococcus* species, cluster A and B, showed a sequence similarity of 94 to 97% to all other *Rhodococcus* spp..

Both clones MAT1-132 (dominant clone) and BT-127 comprised one group within the genus *Dietzia* including four uncultured bacterium clones (sequence similarity of 99%) detected in scum samples collected from a WWTP situated in South Africa (Wagner and Cloete 2002). This group is clearly separated from *Dietzia maris, Dietzia psychralcaliphila* and different *Dietzia* spp. (sequence similarity of 98%). Clones MAT2-218 and BT-117 were grouped within the genera *Gordonia* and *Mycobacterium*, respectively. The sequence of clone MAT-218 branched with *Gordonia spumae* (sequence similarity of 99%). The *Mycobacterium*-related clone BT-117 fell within the group of *M. phlei*, *M. smegmatis*, *M. peregrinum*, and *M. mageritense* but forms an individual branch (sequence similariy of 97 to 98%).

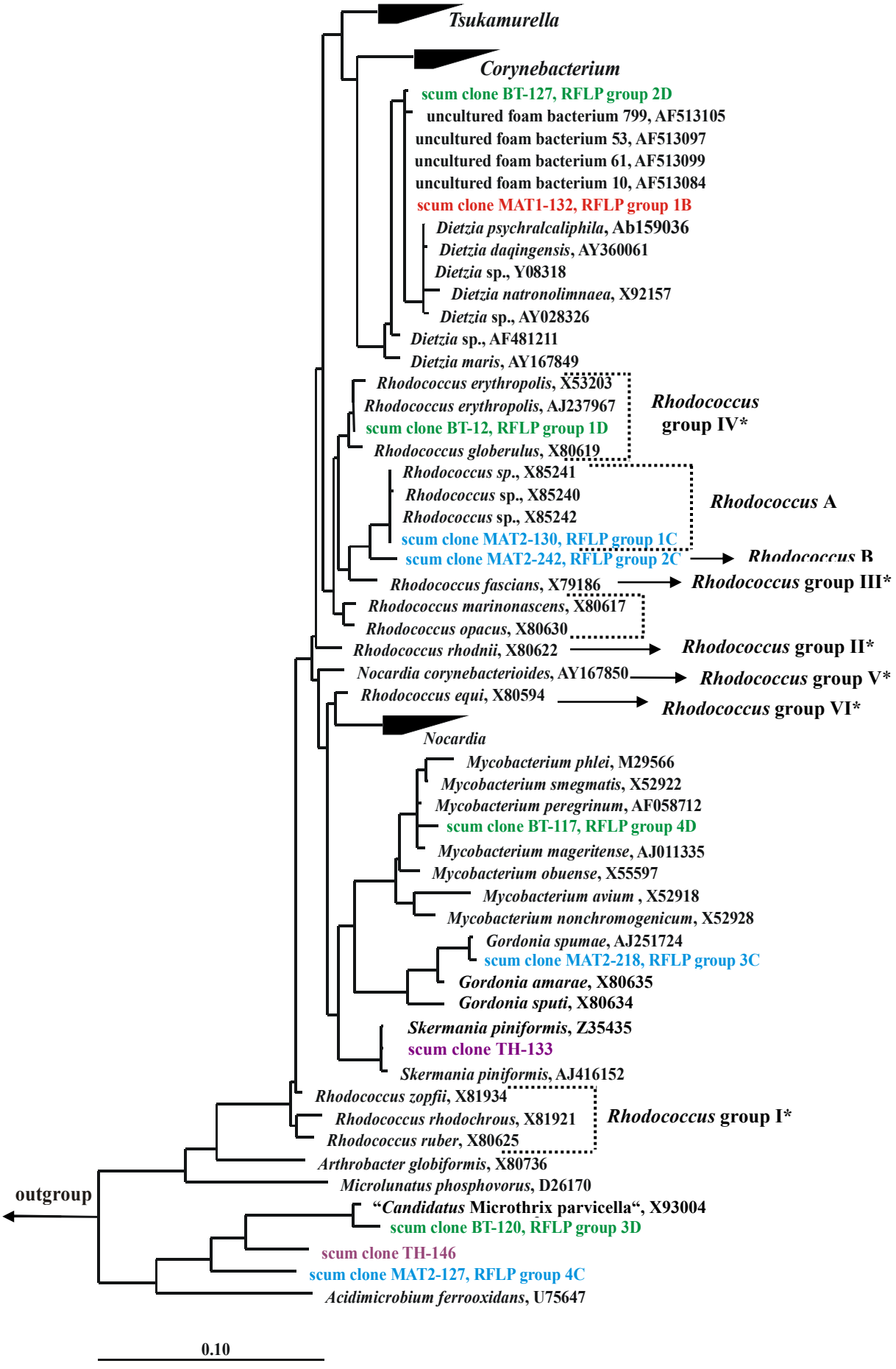

Tsukamurella
Corynebacterium
scum clone BT-127, RFLP group 2D
uncultured foam bacterium 799, AF513105
uncultured foam bacterium 53, AF513097
uncultured foam bacterium 61, AF513099
uncultured foam bacterium 10, AF513084
scum clone MAT1-132, RFLP group 1B
Dietzia psychralcaliphila, Ab159036
Dietzia daqingensis, AY360061
Dietzia sp., Y08318
Dietzia natronolimnaea, X92157
Dietzia sp., AY028326
Dietzia sp., AF481211
Dietzia maris, AY167849
Rhodococcus erythropolis, X53203
Rhodococcus erythropolis, AJ237967
scum clone BT-12, RFLP group 1D
Rhodococcus globerulus, X80619
Rhodococcus group IV*
Rhodococcus sp., X85241
Rhodococcus sp., X85240
Rhodococcus sp., X85242
scum clone MAT2-130, RFLP group 1C
scum clone MAT2-242, RFLP group 2C
Rhodococcus A
Rhodococcus B
Rhodococcus fascians, X79186
Rhodococcus group III*
Rhodococcus marinonascens, X80617
Rhodococcus opacus, X80630
Rhodococcus rhodnii, X80622
Rhodococcus group II*
Nocardia corynebacterioides, AY167850
Rhodococcus group V*
Rhodococcus equi, X80594
Rhodococcus group VI*
Nocardia
Mycobacterium phlei, M29566
Mycobacterium smegmatis, X52922
Mycobacterium peregrinum, AF058712
scum clone BT-117, RFLP group 4D
Mycobacterium mageritense, AJ011335
Mycobacterium obuense, X55597
Mycobacterium avium , X52918
Mycobacterium nonchromogenicum, X52928
Gordonia spumae, AJ251724
scum clone MAT2-218, RFLP group 3C
Gordonia amarae, X80635
Gordonia sputi, X80634
Skermania piniformis, Z35435
scum clone TH-133
Skermania piniformis, AJ416152
Rhodococcus zopfii, X81934
Rhodococcus rhodochrous, X81921
Rhodococcus ruber, X80625
Rhodococcus group I*
Arthrobacter globiformis, X80736
Microlunatus phosphovorus, D26170
outgroup
"Candidatus Microthrix parvicella", X93004
scum clone BT-120, RFLP group 3D
scum clone TH-146
scum clone MAT2-127, RFLP group 4C
Acidimicrobium ferrooxidans, U75647
0.10

Figure 21: Phylogenetic tree of near complete 16S rDNA sequences obtained from uncultured scum bacteria (clone libraries **TH**, **MAT1**, **MAT2,** and **BT)** determined in this study and sequences from publicly accessible databases within the *Actinobacteria* phylum. The tree is based on a maximum likelihood calculation (FastDNAML) and topology was tested by neighbor-joining and maximum parsimony methods (calculation with a 50% positional consensus filter for *Actinobacteria* and a termini filter). The *Firmicutes* group was used as the outgroup in analyses but is not shown in the tree. Multifurications were drawn as a consensus, where the relative branching order could not be determined. The scale bar represents 10% estimated sequence divergence. Members of the six *Rhodococcus* groups of Rainey et al. (1995a) are labeled with a star.

Clone sequence TH-133 clustered within *Skermania piniformis* group with a sequence similarity of > 99%. The tree topology demonstrated that *Skermania piniformis* is building an individual cluster within the nocardioform actinomycetes. Chun et al. (1997) showed that *Skermania piniformis* formed a distinct phylogenetic line most closely related to the genus *Gordonia.* The taxonomic outline of the genera in Bergeys Manual of Systematic Bacteriology (Garrity et al. 2004) affiliated *Skermania piniformis* within the family *Gordoniaceae*. In the present study the tree topologies reconstructed by different mathematical methods demonstrated that the position of *Skermania piniformis* based on 16S rRNA varied. The neighbor joining and maximum parsimony method confirms the close relatedness to the genus *Gordonia* (data not shown). In contrast, the maximum likelihood method, which reproduces the most exact branching (Ludwig and Klenk 2001), indicates that *Skermania piniformis* is related to both genera, *Gordonia* and *Mycobacterium*.

All clone sequences within the *Corynebacterineae* suborder are targeted by the *Actinobacteria*-specific probes HGC1156 and HGC235. The *Dietzia*-related clones MAT1-132 and BT-127, the clone sequence BT-12 classified as *R. erythropolis*, the *Gordonia*-specific clone MAT2-218, and the *Mycobacterium*-specific clone BT-117 matched with both *Corynebacterineae*-specific probes MNP1 and Myc657. In contrast, the *Rhodococcus*-specific sequences placed in cluster A and B showed one and two mismatches to MNP1 and Myc657 probes, respectively. These sequences were only detected with probe CMN119 (Erhart 1997) specific for a subpopulation within the *Corynebacterineae*. Furthermore probe MNP1 failed to target the sequence of the *Skermania piniformis*–related clone TH-133 while no mismatches were detected between the sequence TH-133 and the target sequence of probe Myc657. At genus level the *Gordonia*-related clone sequence (MAT2-218) and the *Skermania piniformis*-related clone sequence (TH-133) matched the probes Gor596 specific for *Gordonia* spp. and NPI425 specific for *Skermania piniformis*, respectively.

5.2.3.6 Design and evaluation of 16S rRNA-targeted oligonucleotide probes for nocardioform actinomycete species

In total, four probes were designed on the basis of the completely sequenced *Dietzia*-related clone, the *R. erythropolis*-classified clone, and the clones grouped within *Rhodococcus* clusters A and B. Probe sequences and their specificity are listed in Table 18. Probe specificities were checked against all publicly available DNA sequences using the BLAST program. Table 19 shows the different alignments of target sequences, matching target organisms, and examples of non–target organisms for the four designed probes calculated using the ARB software program tool probe match. Probe DIE993 detected most of the members within the genus *Dietzia* including the sequences of both clones MAT1-132 and BT-12 and has at least 2 mismatches with non-*Dietzia* species. The sequence divergence within the *Rhodococcus* genus prevents to design a genus-specific probe. Different *Rhodococcus* species were targeted by probes RHOa429, RHOb183, and R.ery619. While probe RHOa429 matched sequences of three *Rhodococcu*s spp. (Soddell et al. 1998) and the clone sequence MAT2-130 within the *Rhodococcus* cluster A, probe RHOb183 targeted only the clone sequence MAT2-242 forming the individual cluster B. Both probes had at least two mismatches to non-targeted organisms. The *R. erythropolis* group including clone sequence BT-12, *R. erythropolis* strains, and some unclassified *Rhodococcus* spp. were detected with probe R.ery619. At least one mismatch to non-targeted organisms was designed for this probe.

Table 18: Probe sequences, target sites, formamide concentrations in the hybridization buffer required for FISH, and specificities of the newly designed probes.

probe	sequence (5′-3′)[a]	target site (16S rRNA position)[b]	formamide [%]	specificity
DIE993	CCGTCGTCCTGTATATGT	993-1010	20	*Dietzia* spp.
RHOa429	CGGAGCTGAAAGGAGTTT	429-446	20	*Rhodococcus* cluster A.
RHOb183	ACCACGAAACATGCATCC	183-200	20	*Rhodococcus* cluster B.
R.ery619	CCTGCAAGCCAGCAGTTG	619-636	20	*Rhodococcus erythropolis*

[a] using oligonucleotide IUB-codes
[b] from E. coli numbering of Brosius et al. (1981)

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Table 19: Sequence alignments showing target sequences, matching target organisms, and examples of non-target organisms for the probes DIE993, RHOa429, RHOb183, and R.ery619 using the ARB software program tool probe match. Capital letters indicate strong mismatches and small letters indicate weak mismatches. Organisms used for evaluation of probe specificities are labeled with gray. Additionally to the accession numbers, DSM numbers (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) are listed in brackets.

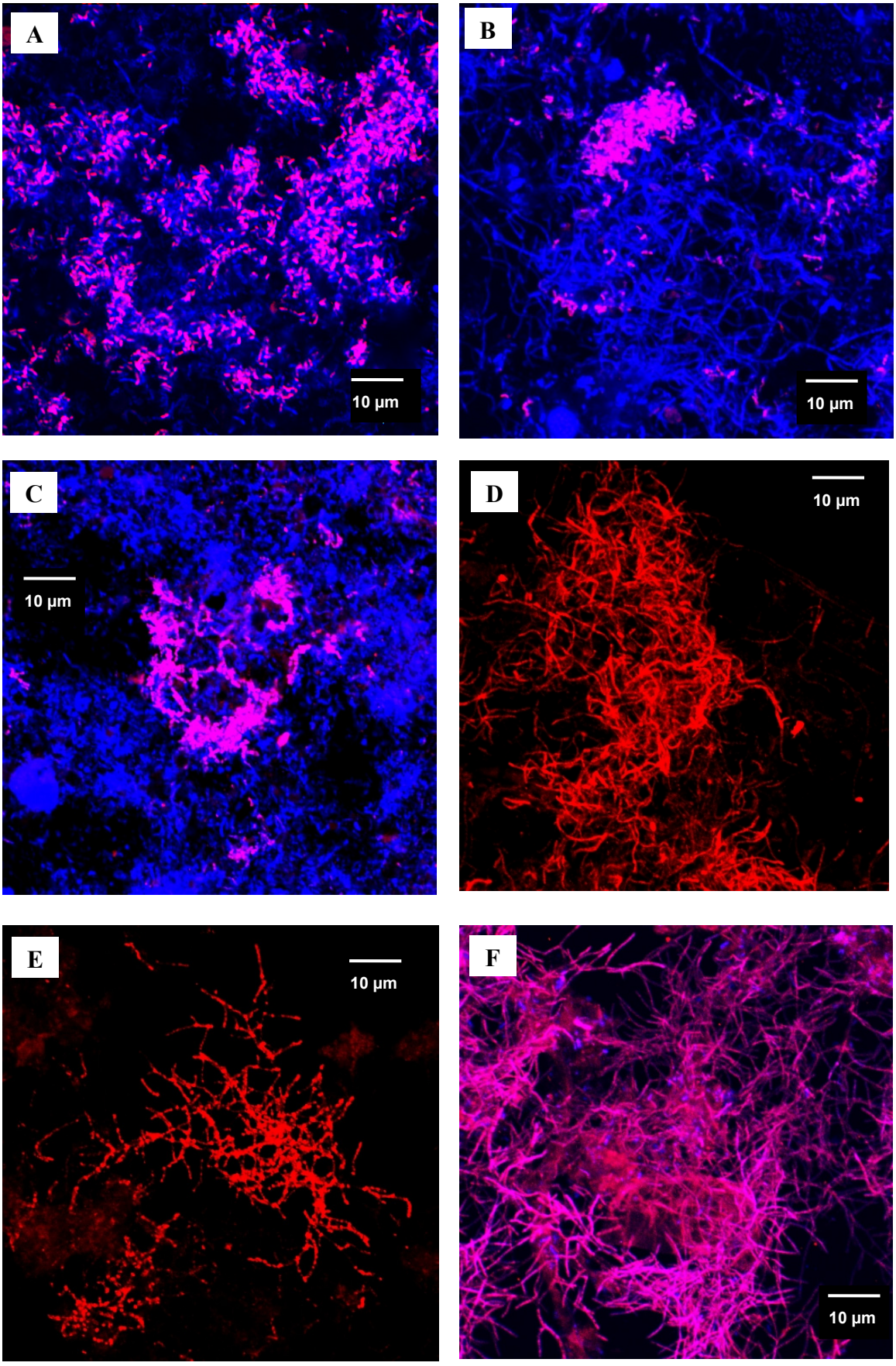
DIE993		**R.ery619**	
probe sequence (3´-5´)	**TGTATATGTCCTGCTGCC**	probe sequence (3´-5´)	**GTTGACGACCGAACGTCC**
target sequence (5´-3´)	**ACAUAUACAGGACGACGG**	target sequence (5´-3´)	**CAACUGCUGGCUUGCAGG**
scum clone MAT1-132	==================	**scum clone BT-12**	==================
scum clone BT-127	==================	*Rhodococcus erythropolis* (X53203/DSM 43188)	=====N==N=========
Dietzia maris (X79290/DSM 43672))	==================	*R. erythropolis* (X76691)	==================
Dietzia psychralcaliphila (AB159036)	==================	*R. erythropolis* (X81929)	==================
Dietzia daqingensis (AY360061)	==================	*R. erythropolis* (AY281114)	==================
Dietzia natronolimnaea (X92157)	==================	*R. erythropolis* (AJ131637)	==================
Dietzia sp. (AY028326)	==================	*R. erythropolis* (AY147846)	==================
Dietzia sp. (AB094465)	==================	*R. erythropolis* (AF420416)	==================
Dietzia sp. (Y08318)	==================	*R. erythropolis* (X80618)	==================
Dietzia sp (Y08313)	==================	*Rhodococcus* sp. (D84420)	==================
Dietzia sp. (Y08312)	==================	*Rhodococcus* sp. (U20791)	==================
uncultured bacterium 10 (AF5113084)	==================	*Rhodococcus* sp. (X9240)	==================
uncultured bacterium 53 (AF5113097)	==================	*Nocardia corynebacterioides* (X80615/ DSM 20151)	======u==========
uncultured bacterium 61 (AF5113099)	==================	*Rhodococcus rhodochrous* (X79288)	=======g==========
uncultured bacterium 799 (AF511105)	==================	*Rhodococcus ruber* (X80625)	=======g==========
Dietzia sp. (AF481211)	==============g===	scum clone MAT2-130	======u==========
Mycobacterium phlei (M29566/DSM43239)	====gC==========	scum clone MAT2-242	======u==========
Rhodococcus coprophilus (X81928)	========C=======U=	*Rhodococcus rhodochrous* (X70295)	=======g==a=======
		Rhodococcus fascians (X81930)	=====U=g==========

RHOa429		**RHOb183**	
probe sequence (3´-5´)	**TTTGAGGAAAGTCGAGGC**	probe sequence (3´-5´)	**CCTACGTACAAAGCACCA**
target sequence (5´-3´)	**AAACUCCUUUCAGCUCCG**	target sequence (5´-3´)	**GGAUGCAUGUUUCGUGGU**
scum clone MAT2-130	==================	**scum clone MAT2-242**	==================
Rhodococcus sp (X85240)	==================	*Mycobacterium bovis*	==========C=u=====
Rhodococcus sp. (X85241)	==================	*Mycobacterium celatum* (L08169/DSM 44243)	==========C=u=====
Rhodococcus sp. (X85242)	==================	*Mycobacterium terrae* (X52925)	===========Cu=====
Gordonia aichiensis (X80633/DSM 439787)	====Cu===========	*Mycobacterium tuberculosis* (X58890)	==========C=u=====
Gordonia sputi (X80634/DSM 43896)	====Cu===========	*Nocardia pseudobrasiliensis* (X84853)	==========C=u=====
Rhodococcus chubuensis (X80627)	====Cu===========	*Rhodococcus ruber* (X80625)	===========C==g===

Pure cultures (see Table 19, gray labeled organisms) of target organisms and non-target organisms (with the fewest mismatches to the probe sequence) and the scum sample MAT2 were used for probe evaluation. Ethanol fixation and lysozyme pre-treatment turned out to be the optimal permeabilization protocol for all pure culture organisms used. The same pre-treatment was applied for the scum sample MAT2 and in addition mild acid hydrolysis in combination with lipase/proteinase K was applied. A range of stringencies with different formamide concentrations (10% increments from 0 to 50%) was applied for each probe. Optimal FISH signals were detected for the target organisms *Dietzia maris* (DSM 43672) and *R. erythropolis* (DSM 43188) using the probes DIE993 and R.ery619, respectively, at 20% formamide. No pure cultures of target organisms were available for probes RHOa429 and RHOb183 but the conspicuous morphology of the target organisms facilitated probe evaluation directly in the original scum sample MAT2. Strong fluorescence signals were determined for both probes using 20% formamide. Applying probes DIE993, R.ery619, RHOa429, and RHOb183 to respective pure cultures of non-target organisms resulted in weak FISH signals at 10% formamide and no signals at 20 % formamide. Therefore the optimal hybridization conditions determined for the probes DIE993, RHOa429, RHOb183, and R.ery619 were 20% formamide

5.2.3.7 FISH with newly designed probes

The newly designed probes were applied to scum samples MAT1, MAT2, and BT that were used for full-cycle 16S rRNA analysis (originated form German WWTPs). Additionally, the scum samples from the Australian WWTP-IN characterized by a NOC-like morphotype and scum samples from both tanks AT1 and AT2 of German WWTP-M from November 2003 were analyzed. The probe DIE993 gave positive FISH signals in all three scum samples MAT1, BT, and IN, detecting NOC-like organisms (Fig. 22 A-C). Additionally to the *Dietzia* spp., *R. erythropolis* growing as branched filaments was present in the scum sample BT as was shown after the application of the R.ery619 probe (Fig. 22 D). The branched filaments present in high numbers in the MAT2 scum (sampling date February 2003) were detectable with the probe RHOb429 specific for the *Rhodococcus* spp. grouped in cluster A (Fig. 22 E). However, merely fragments of the whole filaments were visualized after lysozyme pre-treatment, similar to the results obtained with the *Actinobacteria*-specific probe HGC1156. The application of mild acid hydrolysis in combination with lipase/proteinase K revealed very weak FISH signals. In contrast, probes EUB338-mix and CMN119 detected the whole filaments with stronger signal intensity after the same pre-treatment (Fig. 22 F). The simultaneous hybridization with EUB338-mix indicated that most of the other bacteria cells were lysed after this specific pre-treatment because only the branched filamentous nocardioform actinomycetes gave positive FISH signals. In the same samples only some organisms were detected by probe RHOb183 specific for *Rhodococcus* sp. within cluster B (Fig. 22 G). A higher amount of this organism was present in the scum fractions collected in November 2003 in WWTP-M in both aeration tanks AT1 (Fig. 22 H) and AT2 (Fig. 22 I). In contrast to *Rhodococcus* spp. cluster A, the whole branched filaments of *Rhodococcus* sp. cluster B were visualized after lysozyme treatment of EtOH fixed scum samples by FISH.


A
10 µm
B
10 µm
C
10 µm
D
10 µm
E
10 µm
F
10 µm

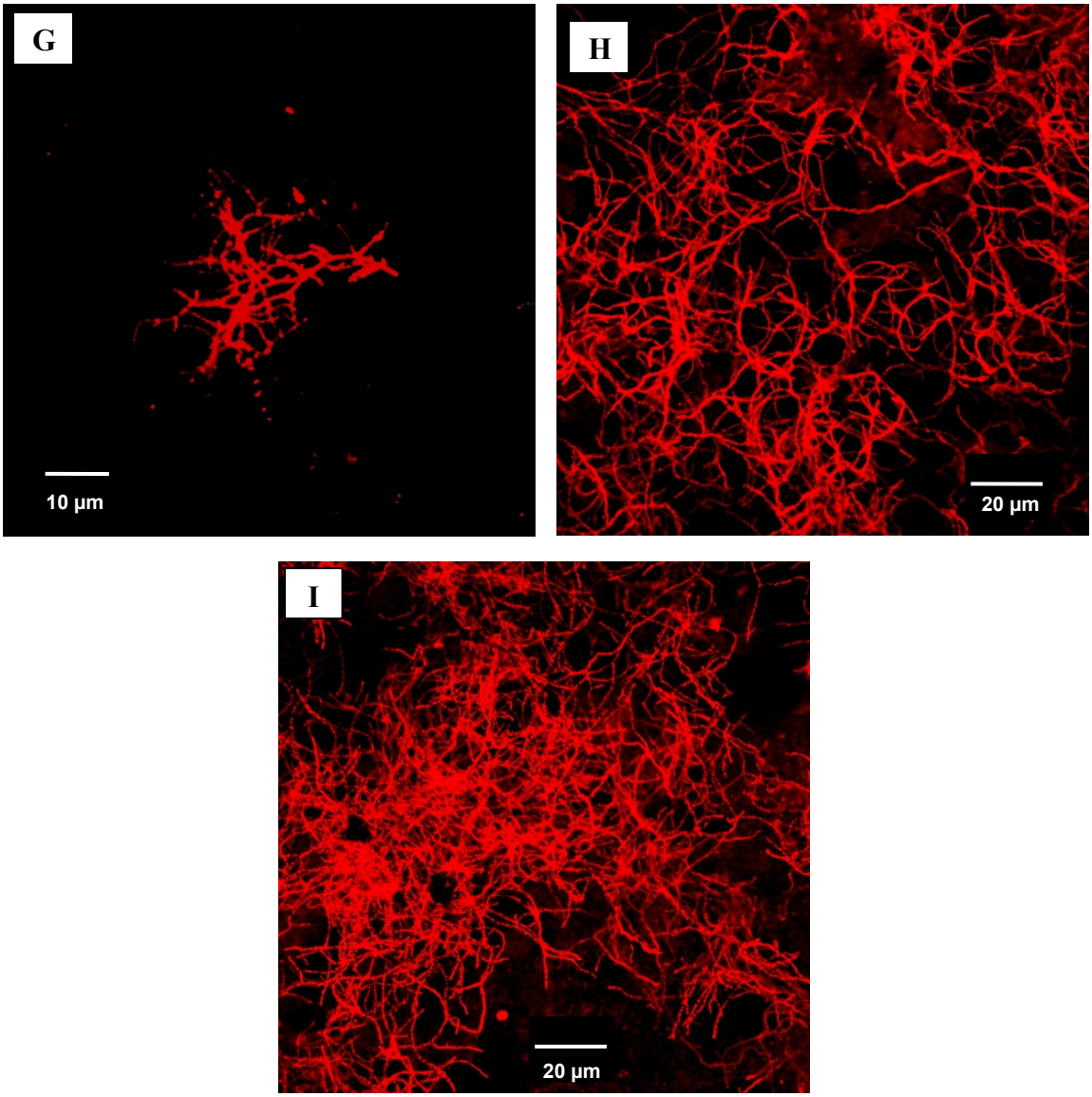


Figure 22: CLSM images after FISH (15 h) using different probes specific for various genera within the *Corynebacterineae* group (nocardioform actinomycetes). ***Dietzia* spp.** (appear magenta) simultaneously hybridized with the CY3-labeled probe DIE993 (red) and CY5-labeled EUB388-mix probes (blue) in different EtOH fixed scum samples: MAT1 (from January 2002, **A**), BT (**B**), and IN (**C**) after lysozyme treatment. **Image D** shows ***R. erythropolis*** developing branched filaments in EtOH fixed scum sample BT after lysozyme treatment and the application of CY3-labeled probe R.ery619. ***Rhodococcus* spp**. **within cluster A** visualized in EtOH fixed scum sample MAT2 (sampling date February 2003) by the CY3-labeled probe RHOa429 after lysozyme treatment (**E**) and the combination of CY3-labeled probe CMN119 (red) and CY5-labeled probes EUB388-mix (blue) after mild acid hydrolysis in combination with lipase/proteinase K, *Rhodococcus* spp. A appear magenta (**F**). Application of CY3-labeled probe RHOb183 specific for ***Rhodococcus* sp. within cluster B** after lysozyme treatment gave positive FISH signals in EtOH fixed scum samples from MAT2 (**G** = sampling date February 2003 and **H** = sampling date November 2003) and MAT1 (**I** = sampling date November 2003).

5.2.4 FISH Quantification

Quantification of nocardioform actinomycetes (*Corynebacterineae*) in the scum fraction of the German WWTPs MAT1, MAT2, and BT and of the Australian WWTPs OX, TH, and IN was carried out by FISH using specific probes for this group, its genera and species (Fig. 23). For all samples lysozyme pre-treatment and 15 h hybridization time were applied. Two different sampling dates were chosen for WWTP-M because the *Rhodococcus* sp. within cluster B was found in quite variable numbers. Bacteria were quantified calculating the percentage area fraction of the specific probe related to EUB338-mix probes in most of the samples. The class index method was used for samples MAT2 collected in February 2003 and TH as here the dominant branched filamentous bacteria present were merely partially detectable by FISH and hence an exact quantification was not possible.

Dietzia spp. were representing approximately 30% of the total bacteria in scum samples originating from WWTP-MAT1 (sampling date January 2002). Furthermore *Dietzia* spp. demonstrated to be the single genus within the detected nocardioform actinomycetes and *Actinobacteria* because comparable percentages were calculated for both the genus *Dietzia* and these groups. *Rhodococcus* spp. grouped within cluster A were detected with a class index of 3.5 applying probe RHOa429 in scum sample MAT2 from February 2003. A comparable class index was determined using the more general probes HGC1156 and CMN119. All three probes detected only fragments of the filaments after lysozyme pre-treatment. Therefore the class index might be higher (class index 4 to 5), which was proved for probes HGC1156 and CMN119 yielding sufficiently strong FISH signals of the whole filaments after mild acid hydrolysis in combination with lipase/proteinase K. Probes DIE993 and RHOb183 visualized very low numbers of organisms in these scum sample (MAT2, February 2003). Despite the *Gordonia*-related clone within the clone library MAT2 in situ analysis applying *Gordonia*-specific probe Gor596 to the original scum samples MAT2 (February 2003) revealed no FISH signals.

A shift within the nocardioform actinomycete population was observed for both tanks MAT1 and MAT2 in November 2003. The dominant organism in both tanks, *Rhodococcus* sp. grouped within cluster B, represented 40% (AT1) and 33% (AT2) of the total bacteria. This organism was detectable with the specific probe RHOb183 and the *Corynebacterineae*-specific probes CMN119 and Myc657, despite the fact that probe Myc657 has two mismatches with the target sequence of this *Rhodococcus* sp.. These observations might be explainable by the fact that according to Davenport et al. (2000) the recommended hybridization conditions detect organisms with one mismatch to the probe sequence. The calculated percentage indicates that probe CMN119 matched only *Rhodococcus* sp.. Probe Myc657 detected additionally other organisms, probably *Dietzia* spp., because these organisms were found with a percentage of 14% and 4% in MAT1 and MAT2, respectively.

Nocardioform actinomycetes within scum BT identified by the *Actinobacteria*-specific probe HGC1156 and the *Corynebacterineae*-specific probe Myc657constituted approximately 40% of the total bacteria. *Rhodococcus erythropolis* was the dominant organism within this group with 28% of the total bacteria. *Dietzia* spp. were identified in lower numbers with 11% of the total bacteria.

In scum samples originating from the both Australian WWTPs, Ox and IN, lower numbers of nocardioform actinomycetes were determined as compared to German scum. *Gordonia* spp. and *Dietzia* spp. were representing 12% and 7% of the total bacteria in the scum samples OX and IN, respectively. These numbers were confirmed by the group-specific probe MNP1. The class index system was used to quantify the filamentous PTLO in the TH scum samples because of the detection problems described above. The dominant organism *Skermania piniformis* was detected with a class index of 3 and might be underestimated because of only partial detection. Additionally *Gordonia* spp. were found with a class index of 2.

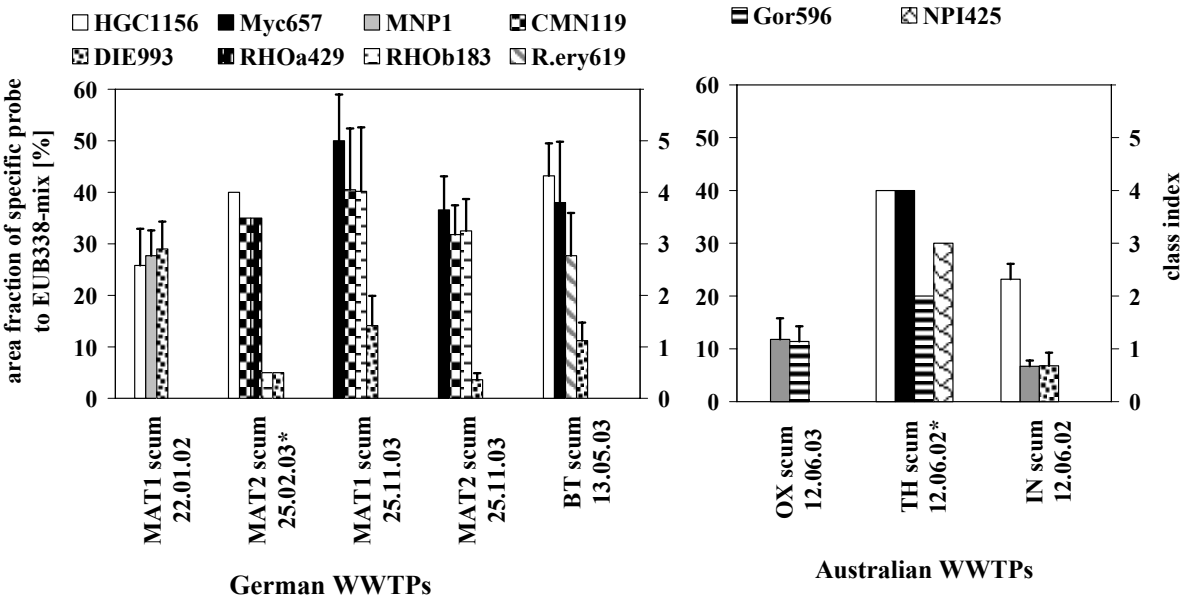


Figure 23: Quantitative FISH results (15 h hybridization time, lysozyme pre-treatment) with several probes calculating the percentage area fraction of the specific organisms related to the domain *Bacteria* (mean value of 15 to 20 images, error bar = corresponding standard deviation) or using class index (samples are labeled with *), carried out on various scum samples originating from different WWTPs (German plants: M, AT1 and AT2, and BT; Australian plants: OX, TH, and IN). HGC1156 specific for *Actinobacteria*; Myc657, MNP1, and CMN119 specific for *Corynebacterineae*; DIE993 specific for *Dietzia* spp.; RHOa429 and RHOb183 specific for *Rhodococcus* spp. of cluster A and cluster B, respectively; R.ery619 specific for *Rhodococcus erythropolis*; Gor596 specific for *Gordonia* spp.; NPI425 specific for *Skermania piniformis*.

5.3 Biological and Physicochemical Characterization

The screening investigations (chapter 5.1) showed that *M. parvicella* and nocardioform actinomycetes are the most frequent organisms found in WWTPs with scum events. On the basis of these results, WWTPs with scum dominated by nocardioform actinomycetes, by *M. parvicella*, and by both nocardioform actinomycetes and *M. parvicella* were chosen for biological and physicochemical characterization. Both activated sludge and scum samples of each WWTP were analyzed at least once in the different seasons. The biological investigations included classical microscopic sludge analysis, FISH quantification of the dominant scum bacteria, and determination of the respiratory activity. Furthermore physicochemical characterizations such as hydrophobicity measurements and EPS analyses from both activated sludge and scum were carried out. In addition, chemical analyses were performed on primary effluent and aeration tank water phase, e.g. secondary effluent. TOC concentration provides information (i) about the total amount of extracted EPS and (ii) the availability of carbon sources for the microbial biocenosis. Furthermore lipophilic substances, carbohydrates, and protein were determined to obtain more detailed knowledge about the kind of carbon source.

5.3.1 Nocardioform Actinomycetes-dominated two-stage WWTP-M

The development of a loose light brown scum layer containing numerous gas bubbles was observed in the aeration tank 1 (AT1) at the surface of the aerated zone from July 2001 to begin of June 2002. This scum fraction was stabilized to a very compact dark brown layer with a very high biomass concentration (50 g VS kg^{-1}) on the surface of the denitrification zone. In June 2002 this layer disappeared and no scum development was observed until the end of November 2003, when scum formation was again detected during a short period of 3 weeks. Scum developed throughout the year 2002 and during winter, early spring, and autumn 2003 on the surface of the aeration tank 2 (AT2). Only during the summer period of 2003 no scum layer was observed on the water surface of AT2. In the middle of September 2003 a stable scum layer was formed again in AT2 and remained there until the end of the sampling period in November 2003. The consistency of the scum fraction in the AT2 varied during the described period. A loose unstable light brown scum layer containing many gas bubbles (15-20 g VS kg^{-1}) was observed from April until November of 2002. In January and November of 2003 a very stable compact scum fraction with a higher biomass concentration (50 g VS kg^{-1}) was formed. Samples were collected from both tanks AT1 and AT2 over the period from April 2002 until November 2003.

The disappearance of the scum layer in AT1 from June 2002 until end of November 2003 can be explained by the operating conditions performed for both tanks. The effluent of AT1 passes to AT2 via a weir, and the surface water and the scum layer of AT2 are blocked by a baffle. End of June 2002 the water level in AT1 was raised that prevented the development of a stable scum layer in AT1 because of scum shifting into AT2. In November 2003 the water level in AT1 was lowered again followed by the formation of a solid brown scum layer on the surface of AT1.

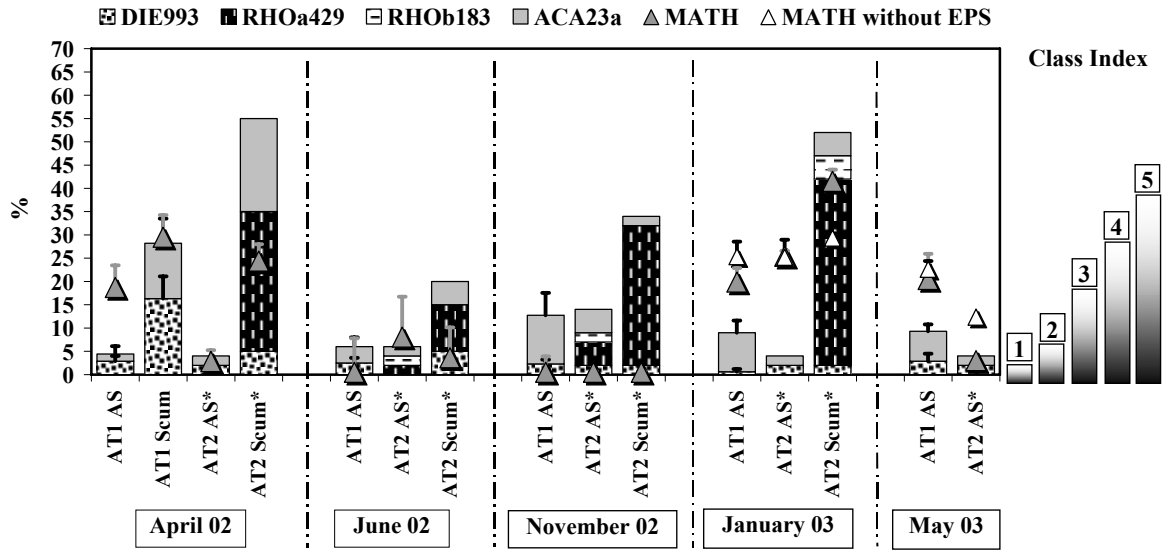
5.3.1.1 Scum bacteria populations and hydrophobicity

Classical microscopic sludge analysis showed that besides nocardioform actinomycetes (GALO and NOC-like organisms) a further scum bacterium, type 1863, was detected in some samples. This filamentous bacterium was identified as *Acinetobacter* spp. by FISH. Additional coccoid shaped *Acinetobacter* spp. cells were detected by FISH. A less frequent filamentous bacterium, type 0041/0675, was found with a class index of 1 to 2 but was never enriched in the scum layer. The application of FISH using the TM7905 probe showed that this bacterium fell within the TM7 division. The dominating nocardioform actinomycetes growing as different morphotypes (NOC-like and GALO) were composed of various genera such as *Dietzia* and two different *Rhodococcus* spp., as was shown by FISH.

FISH quantification was carried out applying the genus- and species-specific probes DIE993, RHOa429, RHOb183, and ACA23a (Fig. 24). In addition, group-specific probes for nocardioform actinomycetes such as Myc657, MNP1, and CMN119 were applied to prove group-, genus-, and species-specific coverage. From April 2002 until May 2003, the class index system was used to quantify dominant organisms in AT2 due to the detection problems of the branched *Rhodococcus* spp.. cluster A, as was described in chapter 5.2, an accurate quantification was not possible. For all other samples the percentage area fraction of the specific organisms related to the area fraction of cells hybridizing with probes for the *Bacteria* domain was calculated.

In contrast to classical microscopy, ***Acinetobacter* spp**. were found in all samples examined by FISH. However, a clear enrichment of *Acinetobacter* spp. in the scum samples of both tanks (MAT1 = 12% and AT2 = class index of 3) was only observed for the sampling date April 2002. Throughout the investigation period from June 2002 until November 2003 the amount of *Acinetobacter* spp. in the activated sludge of MAT1 varied from 1 to 10%. At the end of November 2003 the newly developed scum from AT1 contained very low numbers of *Acinetobacter* spp. cells (3%). In AT2, *Acinetobacter* spp. were only detected in higher numbers in the scum fraction from April 2002 and mid-September 2003 or in activated sludge from November 2002. At this time *Acinetobacter* spp. cells also occurred frequently in AT1. Hence *Acinetobacter* spp. found in AT2 sludge might have originated from AT1 and entered AT2 by sludge shifting. *Acinetobacter* spp. usually grew as filaments or accumulated to large cell clusters explaining the high standard deviation often calculated for this organism.

Nocardioform actinomycetes were detected in all samples analyzed. Low numbers were determined in activated sludge (AT1 from April 2002 until Nov. 2003: Myc657 detected < 1 to 9% ot the total bacteria, AT2 from April 2002 until May 2003: class index of 0.5 to 1 was detected with probe CMN119, AT2 from Sept. 2003 until Nov. 2003: Myc657 detected 1 to 3% of the total bacteria). Significantly higher numbers were found in scum samples with a percentage of area fraction of 15 to 54% (+/- 2 to 9%, AT1 from April 2002 and Nov. 2003, and AT2 from Sept. 2003 and Nov. 2003) and a class index of 2 to 5 (AT2, April 2002 until May 2003) detected with probes Myc657 and CMN119, respectively. The comparison of these results with the detection frequency on genus and species level (Fig. 24) demonstrated that the specific probes applied identified the nocardioform actinomycete species in the scum samples analyzed. The application of probe DIE993 showed that *Dietzia* spp. present in most of the samples was detected as sole nocardioform actinomycete in AT1 scum samples with quite a high amount of 16% in April 2002 only.



***class index, AT2**

probes	April 02		June 02		November 02		January 03		May 03
	AS	scum	AS	Scum	AS	scum	AS	scum	AS
DIE993	0.5	1.5	0	1	0.5	0.5	0.5	0.5	0.5
RHOa429	0	4	0.5	2	1	4	0	5	0
Rhob183	0	0	0.5	0	0.5	0	0	1	0
ACA23a	0.5	3	0.5	1	1.5	0.5	0.5	1	0.5

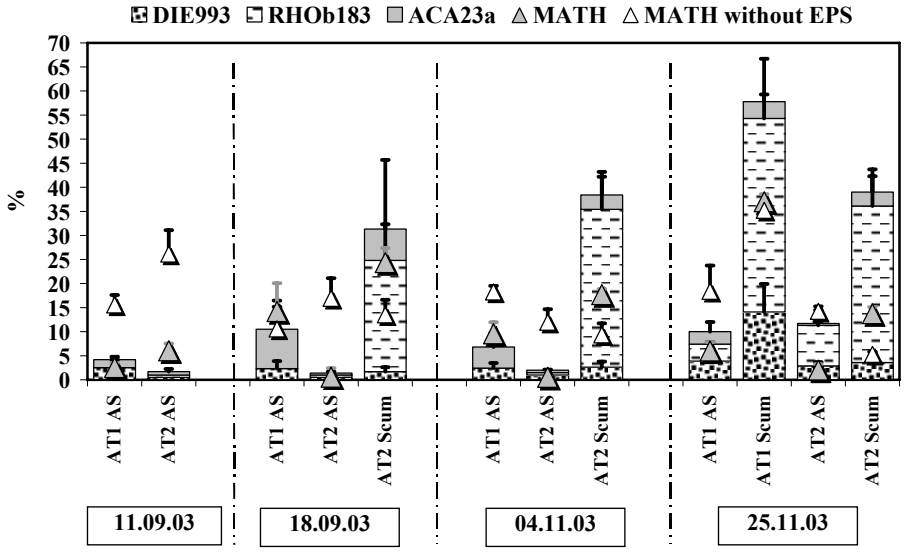


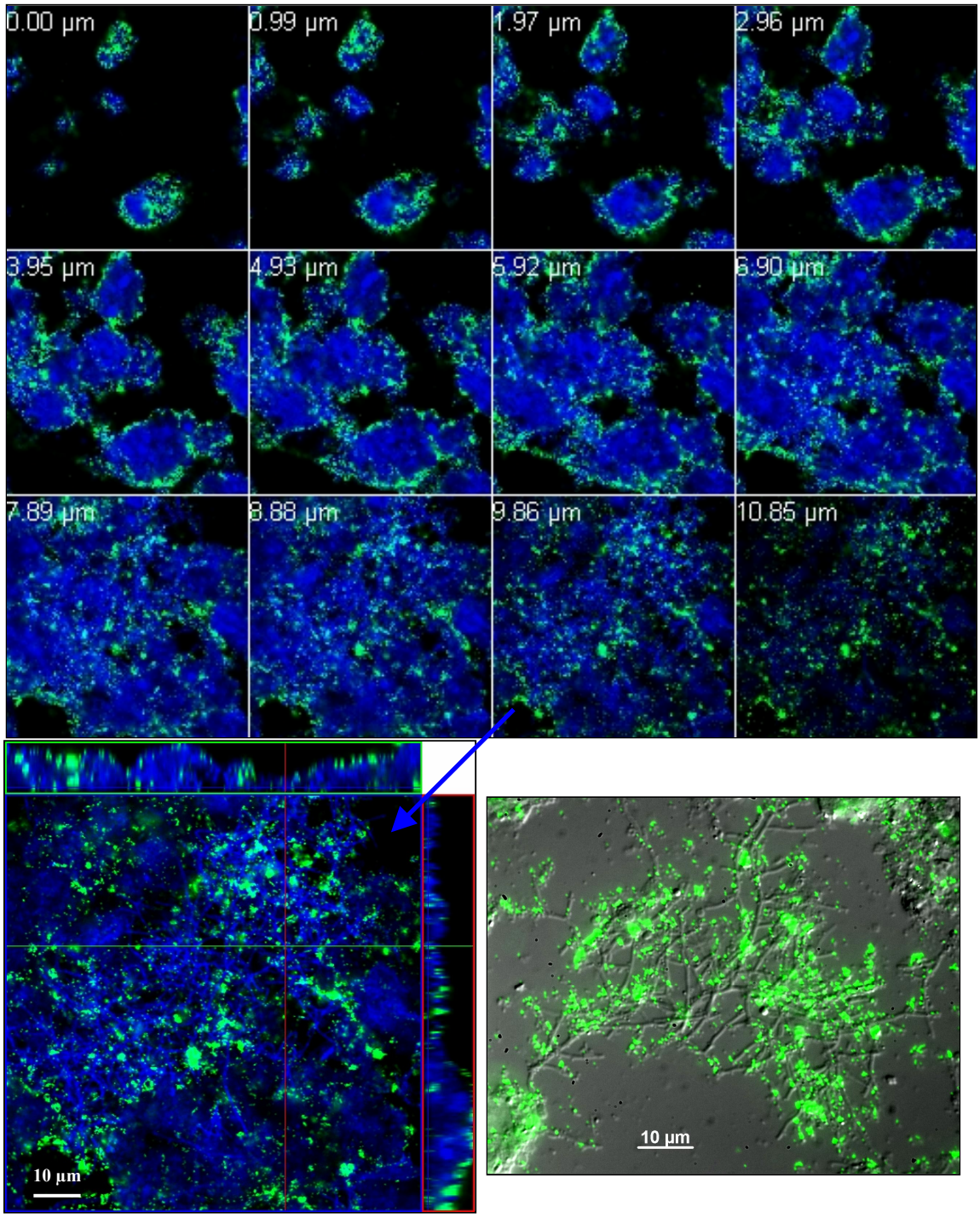
Figure 24: Comparison of **quantitative FISH** results of dominant scum bacteria (nocardioform actinomycetes: 15 h hybridization time, lysozyme pre-treatment) calculated by percentage area fraction of the specific probes related to the EUB338-mix (mean value of 15 to 30 images, error bar = corresponding standard deviation) or by class index (samples are labeled with a star) and **hydrophobicity** (MATH in %, mean value and error bar = standard deviation calculated from 5 replicates). Activated sludge (AS) and scum samples originating from WWTP-M, AT1 (= high load stage) and AT2 (= low load stage). Specificity of probes: **DIE993** = *Dietzia* spp.; **RHOa429** = *Rhodococcus* spp. cluster A; **RHOb183** = *Rhodococcus* sp. cluster B; **ACA23a** = *Acinetobacter* spp..

AT1 scum samples from November 2003 and AT2 scum samples were dominated by *Rhodococcus* spp.. They were clearly enriched in the scum fraction as compared to the corresponding activated sludge. From April 2002 until January 2003 *Rhodococcus* spp. cluster A detected by probe RHOa429 were quantified with a class index of 2 to 5 in scum from AT2. A decline of these organisms was observed from April 2002 until June 2002 and a continuous increase was found from June 2002 until January 2003 with the highest class index of 5. After the summer season of 2003 where no scum formation was observed a different *Rhodococcus* sp. (cluster B) targeted by probe RHOb183 was detected in the scum samples from AT2. This *Rhodococcus* sp. was found with an area fraction of 20 to 30% in all scum samples from AT2 during the autumn season of 2003. At the end of November this organism was present also in very high numbers (40%) in a one-week-old scum layer floating on the water surface from AT1. In addition to the dominant *Rhodococcus* sp., *Dietzia* spp. were found to be clearly enriched in this scum fraction from AT1 as compared to activated sludge with an area fraction of 14%.

The hydrophobicity data determined by the MATH-test (Microbial Adhesion To Hydrocarbons, Fig. 24) increase in many cases but not all with the frequency of nocardioform actinomycetes and *Acinetobacter* spp.. In November 2002 both activated sludge (AT1 and AT2) and scum (AT2) showed no detectable hydrophobic properties. In a further experiment for some samples hydrophobicity was measured after EPS extraction to show in more detail the effect of EPS. Activated sludge from both tanks showed mostly an increase of hydrophobicity after EPS extraction indicating that the extraction procedure released hydrophobic cell surfaces and hydrophobic areas within the sludge flocs. For some activated sludge samples and the scum fraction from AT1 no differences were determined before and after EPS extraction. In contrast, a decrease of approximately 10% hydrophobicity was detected in all scum samples from AT2 after EPS extraction demonstrating scum EPS cause hydrophobic properties.

In situ analyses of hydrophobicity by the MAC method (Microsphere Adhesion to Cells) showed, in particular for scum from AT2, the hydrophobic fluorescent microspheres preferably to attach to the edge of sludge flocs. Inside the flocs they were detected only if the floc structure was open (Fig. 25). The reason for these observations might be diffusion limitations of the microspheres. Focusing on the cell surface of nocardioform actinomycetes it could be demonstrated that the GALO morphotype identified as different *Rhodococcus* spp., which were found in scum from AT2 (January 2003 and November 2003), merely partially attracted hydrophobic microspheres (Fig. 25).

Figure 25: CLSM images show a scum sample from MAT2 (January 2003) after the application of nucleic acid dye Syto60 (cells appear blue) and green fluorescent hydrophobic microspheres, gallery of 12 sections (Δz = 1 µm) and single image of section 11 with corresponding z-scans. Epifluorescence image superimposed with phase contrast image shows morphotype GALO in scum from MAT2 (January 2003) after application of green fluorescent hydrophobic microspheres.



5.3.1.2 EPS characterization

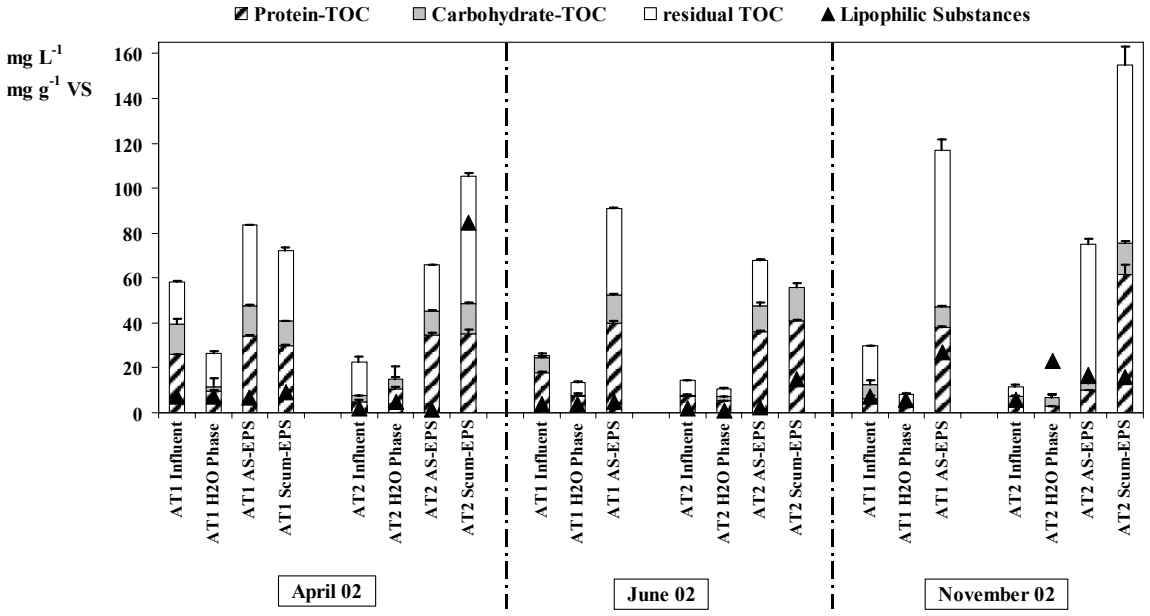
TOC concentrations (Fig. 26) of the influent AT1 and AT2 reflect the operating conditions for each stage with generally higher values for AT1 (20 to 70 mg L^{-1}) as compared to AT2 (10 to 20 mg L^{-1}). In both tanks the water phase usually showed low TOC values of 10 to 20 mg L^{-1}. In the EPS fraction a TOC amount of 60 to 150 mg g^{-1} VS was analyzed with the highest values in scum originating from AT2, November 2002. In AT1 only slight differences in the TOC content of EPS extracted from activated sludge and scum were determined in April 2002. In contrast, a higher TOC value in EPS of scum as compared to activated sludge was

analyzed at the end of November 2003. Furthermore in general a higher TOC concentration was analyzed in the EPS fraction extracted from the activated sludge of AT1 in comparison with activated sludge of AT2. However, in most cases the extracted scum EPS from AT2 yielded significantly higher TOC values as compared to both activated sludge samples. The lowest TOC concentration in scum EPS was determined in the AT2 at June 2002 correlating positively with the slow growth rate of nocardioform actinomycetes in this tank.

The total protein and carbohydrate content were calculated in TOC units to obtain the fraction of TOC (Fig. 26). The protein content in the influent of AT1 was significantly higher (7 to 25 mg L^{-1}) as compared to the influent of AT2 (4 to 8 mg L^{-1}). A similar amount of protein in a range of 2 to 10 mg L^{-1} was usually detected in the water phase of both tanks. The EPS of both activated sludge and scum showed concentrations of 20 to 40 mg g^{-1} VS. A significantly higher concentration of protein in the scum fraction in comparison to activated sludge was detected in November 2002 in AT2 and at the end of November 2003 in AT1. Very low protein amounts in the EPS of activated sludge of both tanks were measured at the beginning of September 2003 one week before scum formation was observed again in AT2. Carbohydrates were found in significantly lower concentrations as compared to protein. For most of the examined EPS samples a protein to carbohydrate ratio of 2 to 6 was calculated.

In most of the samples analyzed, particularly the EPS samples, the components protein and carbohydrate represent only 30 to 60% of the TOC. The residual fraction of TOC might include lipophilic substances or other constituents, e.g. volatile fatty acids, alcohols, amino acids, and detergents, found in municipal wastewater. In the current measurements only lipophilic substances being supposed to influence scum formation were analyzed. They were detected in most samples at a low concentration (Fig. 26). Only once they revealed to be distinctly enriched in the scum EPS from AT2 in April 2002 with an amount of 85 mg g^{-1} VS, i.e. 40fold higher concentration of lipophilic substances as compared to activated sludge EPS. In November 2002, activated sludge EPS from both tanks and the scum EPS from AT2 showed values of lipophilic substances between 16 and 27 mg g^{-1} VS with the highest content in the activated sludge EPS from AT1. A similar amount of lipophilic substances were detected in January 2003 but with an enrichment in scum EPS from AT2. The influent values of AT1 in January, May and November 2003 indicate lipophilic substances to probably enter the system by the primary effluent as the WWTP from time to time faces problems with the grease separator. The generally low concentration of lipophilic substances in the primary effluent water phase might be due to particulate lipid flotation as was corroborated by the high values of lipophilic substances of about 2 to 9 g L^{-1} being determined in water samples taken from the tank surface. The reason for the accumulation of the lipophilic substances at the surface of the AT2 is probably that the effluent of AT1 passes to AT2 via a weir and the surface water of AT2 is blocked by a baffle.

Figure 26: TOC (mean value and error bar = standard deviation calculated from 2 replicates), carbohydrate and protein content (determined as TOC units, mean value and error bar = standard deviation calculated from 3 replicates), and lipophilic substances in the primary effluent [mg L^{-1}], aeration tank water phase [mg L^{-1}], and EPS [mg g^{-1} VS] extracted from activated sludge (AS) and scum from WWTP-M, AT1 = high load stage and AT2 = low load stage.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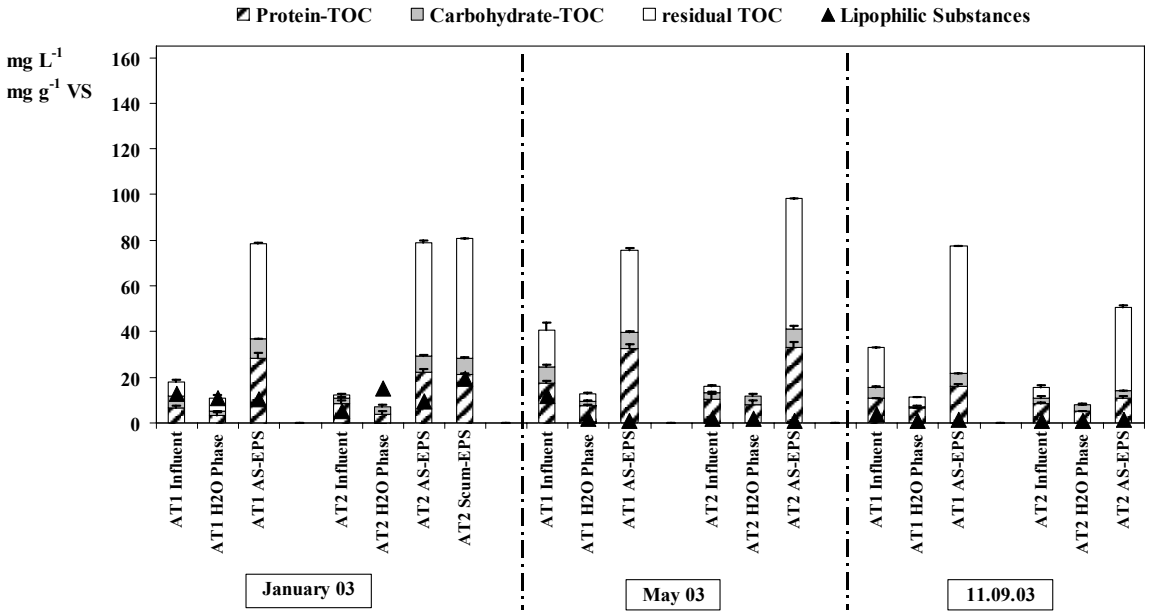
Carbohydrate-TOC
residual TOC
Lipophilic Substances
mg L-1
mg g-1 VS
160
140
120
100
80
60
40
20
0
AT1 Influent
AT1 H2O Phase
AT1 AS-EPS
AT1 Scum-EPS
AT2 Influent
AT2 H2O Phase
AT2 AS-EPS
AT2 Scum-EPS
April 02
June 02
November 02


Protein-TOC
Carbohydrate-TOC
residual TOC
Lipophilic Substances
mg L-1
mg g-1 VS
160
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120
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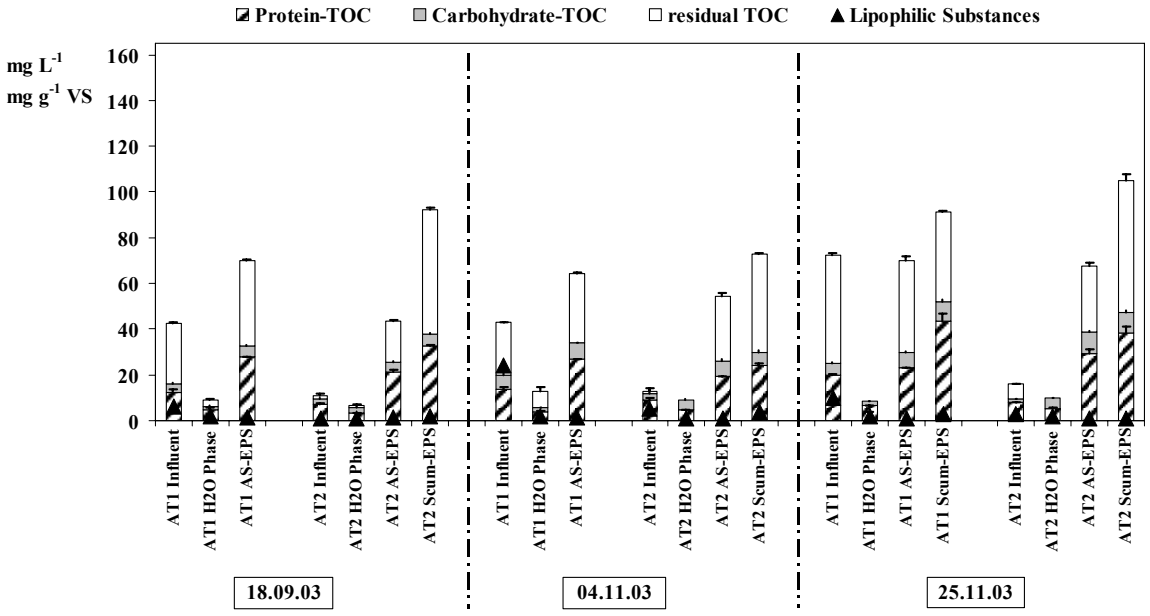
80
60
40
20
0
AT1 Influent
AT1 H2O Phase
AT1 AS-EPS
AT2 Influent
AT2 H2O Phase
AT2 AS-EPS
AT2 Scum-EPS
January 03
May 03
11.09.03


Protein-TOC
Carbohydrate-TOC
residual TOC
Lipophilic Substances
mg L-1
mg g-1 VS
160
140
120
100
80
60
40
20
0
AT1 Influent
AT1 H2O Phase
AT1 AS-EPS
AT1 Scum-EPS
AT2 Influent
AT2 H2O Phase
AT2 AS-EPS
AT2 Scum-EPS
18.09.03
04.11.03
25.11.03

5.3.2 *Microthrix parvicella*-dominated WWTP-GR

Throughout the entire investigation period from January 2003 until November 2003 a stable brown scum layer floated on the water surface of the aeration tank from WWTP-GR. A ten time higher biomass concentration of 30 to 35 g VS kg^{-1} was determined in the scum fraction as compared to activated sludge.

5.3.2.1 Scum bacteria populations and hydrophobicity

The scum bacterium *M. parvicella* was detected frequently in activated sludge and scum and identified as "*Candidatus* M. parvicella" by FISH. Filamentous types 0092, 0041/0675, 1851, *N. limicola*, and NOC-like organisms were found usually in distinctly lower numbers with a class index of 1 to 2 in activated sludge. In contrast to *M. parvicella*, they were never enriched in the scum fraction. Type 0092 occurred in higher numbers with a class index of 3 during summer only. A population shift of the other filamentous bacteria was not observed. The application of FISH using specific probes classified type 0041/0675 within the TM7 division, type 1851 within the *Chloroflexi* phylum, *N. limicola* as "*Candidatus* N. limicola" belonging to the *Actinobacteria* phylum, and NOC-like organisms as *Dietzia* spp.. No taxonomic information was obtained for type 0092.

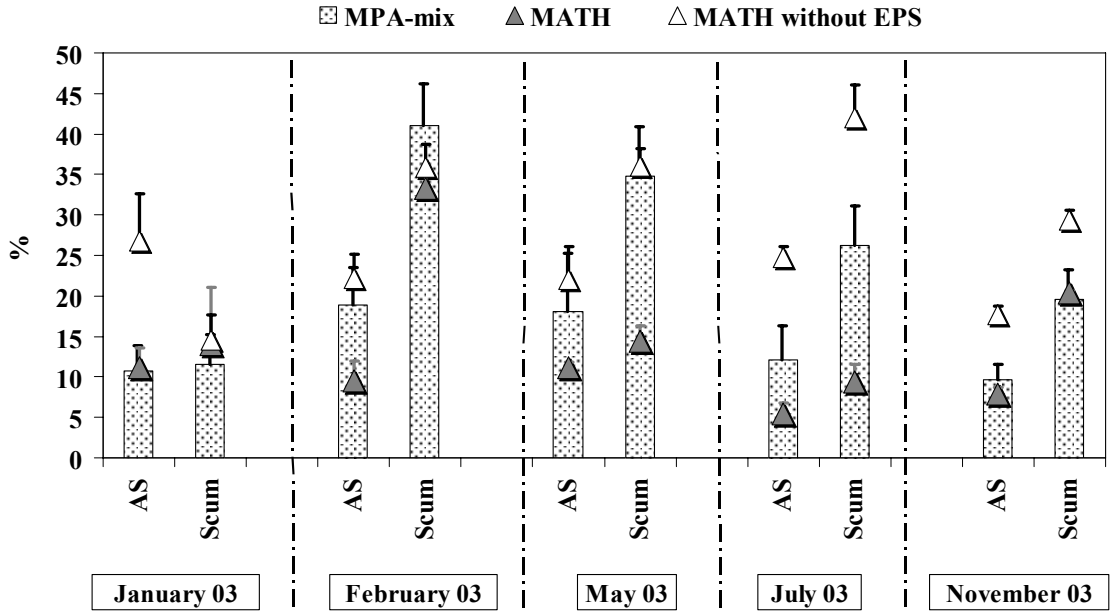


Figure 27: **FISH quantification** (15 h hybridization time, lysozyme pre-treatment) shows percentage area fraction of the specific probes detecting "*Candidatus* M. parvicella" (**MPA-mix** = MPA60, MPA223, MPA645) related to the EUB338-mix detecting the domain *Bacteria* (mean value of 20 to 30 images, error bar = corresponding standard deviation). **Hydrophobicity** determined as MATH in % (mean value and error bar = standard deviation calculated from 5 replicates). Activated sludge (AS) and scum samples originating from WWTP-GR.

Figure 27 shows the quantification results for *M. parvicella* and the MATH values in both activated sludge and scum over the sampling period 2003. In January a similar area fraction of 11 to 12% was calculated for both activated sludge and scum. In February slightly higher numbers of 19% were detected in activated sludge whereas a significant increase to 41% was

observed in scum. A continuous reduction of *M. parvicella* was determined in the scum fraction from February to November. Nevertheless *M. parvicella* was still present in very high numbers during summer and autumn with 10 to 12% and 20 to 26% in activated sludge and scum, respectively.

In February and November the MATH-test revealed distinctly higher values in the scum samples (20 to 33%) as compared to activated sludge (8 to 10%) corresponding to the increase of *M. parvicella* filaments in scum. In contrast, hydrophobicity was low in both activated sludge and scum from May and July (5 to 14%) despite high numbers of *M. parvicella*. The MATH-test with samples after EPS extraction caused a significant increase of hydrophobicity of most samples, which was most distinct in May and July 2003. Therefore it was hypothesized that (i) the filamentous bacterium *M. parvicella* had similar hydrophobic cell surfaces during the whole sampling period and (ii) *M. parvicella* due to its hydrophobic cell surface might influence hydrophobic sludge properties. The lower MATH-values before the EPS extraction determined for some samples point out that *M. parvicella* grew preferably inside the flocs as was confirmed by microscopic analysis and was consequently not detected by the MATH-test.

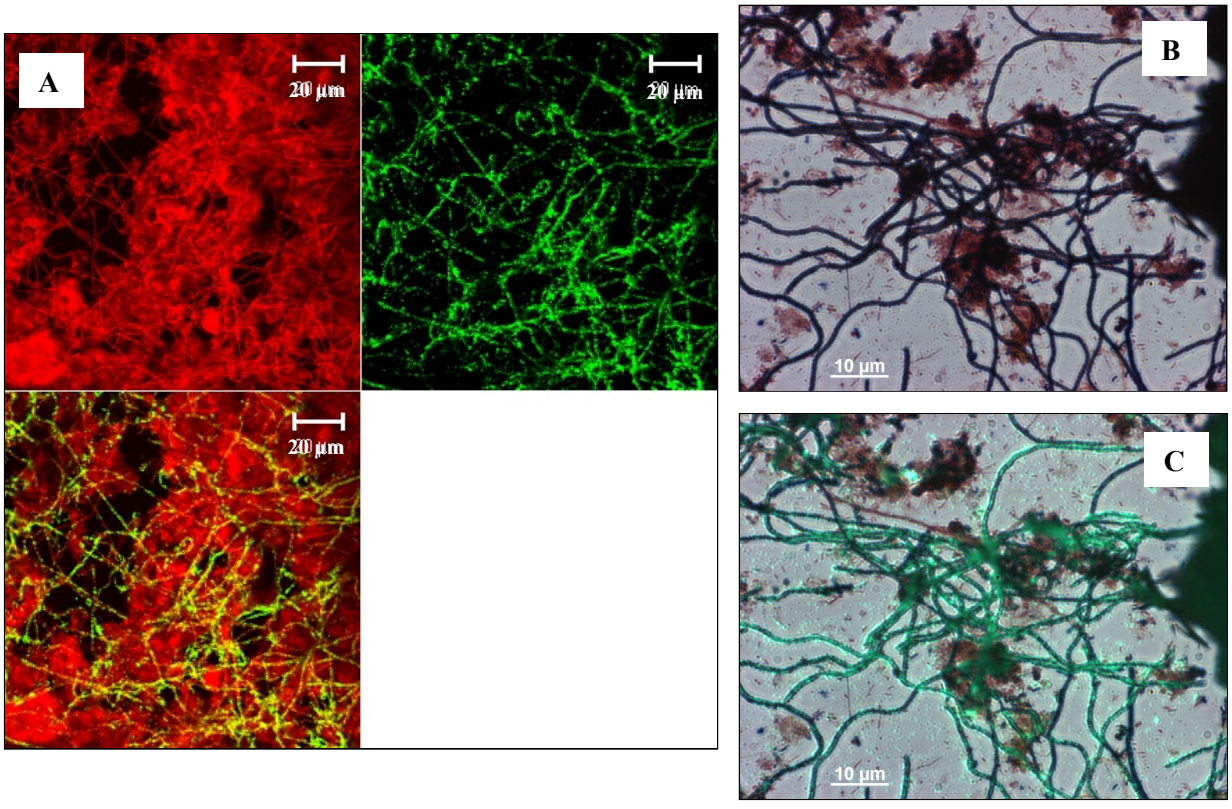


Figure 28: Single CLSM images visualize bacteria in scum (WWTP-GR, January 2003) after application of nucleic acid dye Syto60 (cells appear red) and green fluorescent hydrophobic microspheres (**A**). Bright field images show filamentous *M. parvicella* after Gram staining (**B**). Bright field superimposed with epifluorescence image (**C**) demonstrates green fluorescent hydrophobic microspheres to be attached to the surface of *M. parvicella* in scum samples originating from WWTP-GR (January 2003).

Furthermore an in situ analysis of the hydrophobic properties of activated sludge and scum by the MAC-method was carried out. In contrast to the samples originating from WWTP-M, in general no attachment of hydrophobic microspheres to the flocs was observed. Only the cell surface of the filamentous bacterium identified as *M. parvicella* after Gram staining was covered by fluorescent hydrophobic microspheres in all samples analyzed (Fig. 28). These observations confirm this scum organism to cause hydrophobicity of activated sludge and scum by its cell surface properties.

5.3.2.2 EPS characterization

In the *M. parvicella*-dominated WWTP-GR TOC values of 33 to 72 mg g^{-1} VS were analyzed in the EPS from activated sludge and scum (Fig. 29). These values are distinctly lower than those from WWTP-M. The TOC content in the primary effluent fluctuated from 25 to 140 mg L^{-1} over the investigation period. The aeration tank water phase yielded very low TOC values (7 to 14 mg L^{-1}) during winter and autumn with an increase in spring and summer (43 to 46 mg L^{-1}). In July the high TOC values in the aeration tank water phase were accompanied with distinctly lower TOC concentrations in the activated sludge EPS as compared to other sampling dates.

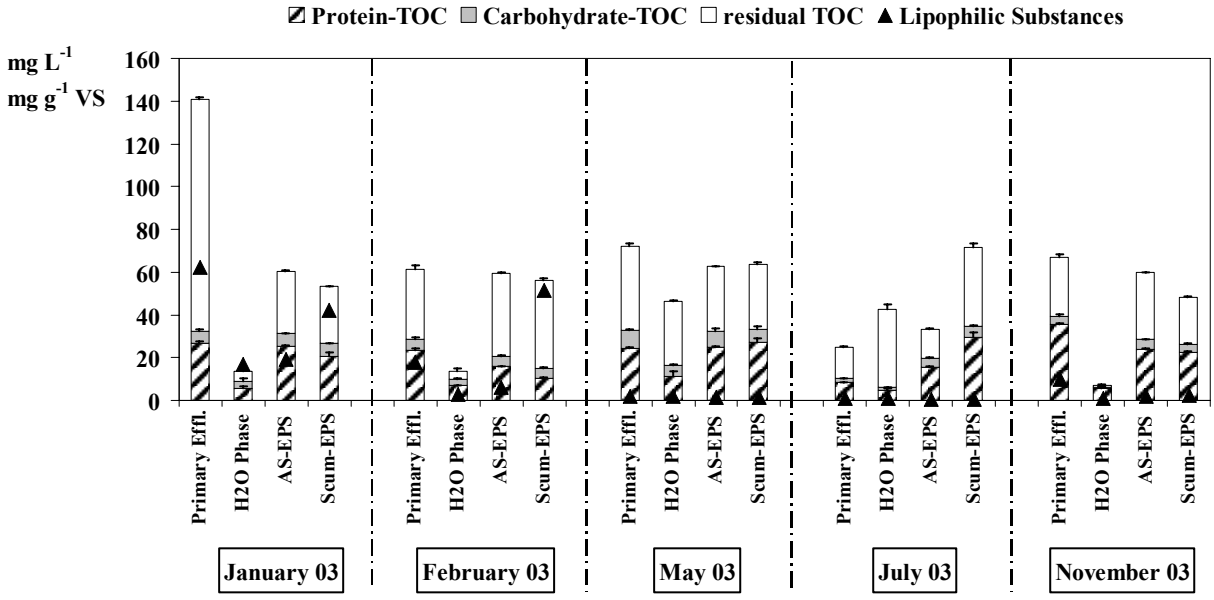


Figure 29: TOC (mean value and error bar = standard deviation from 2 replicates), carbohydrate and protein content (determined as TOC units, mean value and error bar = standard deviation calculated from 3 replicates), and lipophilic substances in the primary effluent [mg L^{-1}], aeration tank water phase [mg L^{-1}], and EPS [mg g^{-1} VS] extracted from activated sludge (AS) and scum of WWTP-GR.

No noticeable changes in the amounts of protein (23 to 26 mg L^{-1}) and carbohydrate (5 to 8 mg L^{-1}), both given in TOC concentrations, were determined in the primary effluent over the sampling period from January to May(Fig. 29). A distinctly lower protein concentration was determined in July and a significantly higher protein content in November. In the aeration tank water phase, low values of protein (5 to 11 mg L^{-1}) and carbohydrate (1 to 5 mg L^{-1}) were analyzed despite the detected higher values in the primary effluent. A protein content in a range of 10 to 30 mg g^{-1} VS and a carbohydrate content in a range of 4 to 8 mg g^{-1} VS were

found in the EPS of activated sludge and scum. In comparison to WWTP-M, lower concentrations of protein and carbohydrate in the EPS were found in the WWTP-GR; however, protein to carbohydrate ratios of 2 to 6 were similar.

The residual fraction of TOC that was not characterized by protein and carbohydrate measurements amount 15 to 108 mg L^{-1} in the primary effluent, 1 to 36 mg L^{-1} in the water phase and 14 to 41 mg g^{-1} VS within the EPS samples. During the winter season lipophilic substances might represent these fractions (Fig. 29). High values of lipophilic substances (62 mg L^{-1}) were measured in the primary effluent in January. In the aeration tank water phase, the EPS of activated sludge and scum concentrations of 17 mg L^{-1}, 19 mg g^{-1} VS and 42 mg g^{-1} VS, respectively, were detected. In February a significantly lower amount of lipophilic substances (18 mg L^{-1}) entered the system. This caused a decline of the lipophilic substances in the aeration tank water phase and the activated sludge EPS. In contrast, scum EPS were still characterized by a high fraction of lipophilic substances of 52 mg g^{-1} VS. From May to November aeration tank water phase and EPS showed very low values of lipophilic substances (1 to 2 mg L^{-1} and 1 to 2 mg g^{-1} VS, respectively). Comparably low concentrations of lipophilic substances were also determined for the primary effluent in May and July. An increase in the primary effluent was observed in November (10 mg L^{-1}).

5.3.3 Nocardioform Actinomycetes- and *Microthrix parvicella*-dominated WWTP-BT

During the sampling period from May 2003 until February 2004 the formation of a stable solid brown scum layer was observed on the water surface of the aeration tank from WWTP-BT. A high biomass concentration of 50 g VS kg^{-1} was determined for the scum fraction.

5.3.3.1 Scum bacteria populations and hydrophobicity

A significant shift of the dominant scum bacteria, nocardioform actinomycetes (GALO and NOC-like organims) to *M. parvicella,* was observed in July in WWTP-BT. Other filamentous bacteria such as types 0092, 0041/0675 (TM7 division), 1851 (*Chloroflexi* phylum), and *N. limicola* ("*Candidatus* N. limicola", *Actinobacteria* phylum) occurred in low numbers with a class index of 1. Population shifts of these organisms were not detected during the observation period.

FISH quantification was carried out for the dominant scum bacteria identified as "*Candidatus* M. parvicella", *Rhodococcus erythropolis*, and *Dietzia* spp. (Fig. 30). *M. parvicella* was detected frequently in July 2003 with high numbers in activated sludge (26%) being clearly enriched in the scum layer (48%). Simultaneously a very low amount of nocardioform actinomycetes was detected by probe Myc657 (2% and 5%). In contrast, *M. parvicella* was found in distinctly lower numbers in activated sludge (3 to 5%) and scum (5 to 13%) during the remaining sampling dates. In these scum samples nocardioform actinomycetes appeared frequently with the highest detection frequency in May 2003 (38%) and the lowest in November 2003 (17%). In the corresponding activated sludge nocardioform actinomycetes occurred in distinct lower numbers representing 3 to 8% of the total bacteria. In all scum samples *R. erythropolis* and *Dietzia* spp. seemed to represent the only nocardioform actinomycete species. However, the frequency of these organisms differed. In May 2003 and February 2004 the dominant organism was *R. erythroplis* detected with 28% and 29% of the total bacteria, respectively. At the same time *Dietzia* spp. were found with 11% in May and

only 4% in February. In November 2003 both organisms were present with lower percentages of 5 (*R. erythropolis*) and 8 (*Dietzia* spp.). In contrast to the scum population, most of the nocardioform actinomycetes in activated sludge identified by probe Myc657 grew as single rods and were not targeted by the *Dietzia*- and *R. erythropolis*-specific probes.

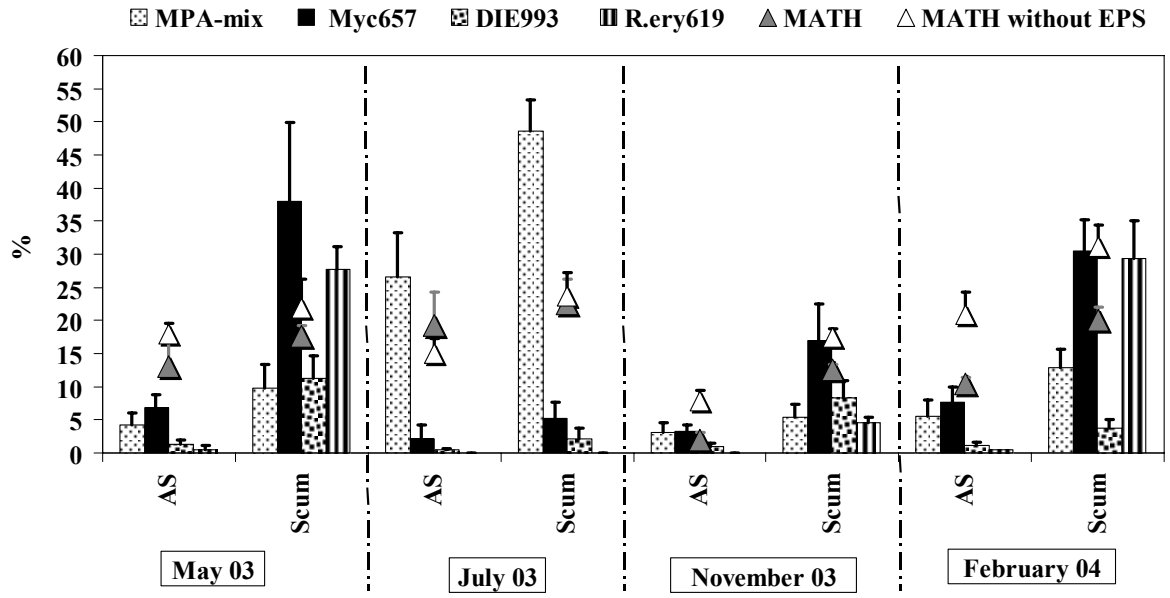


Figure 30: **FISH quantification** (15 h hybridization time, lysozyme pre-treatment) of the dominant scum bacteria in activated sludge (AS) and scum from WWTP-BT. Percentage area fractions of a specific probe related to EUB338-mix detecting the domain *Bacteria* were calculated (mean value of 15 to 20 images, error bar = corresponding standard deviation). Specificity of applied probes: **MPA-mix** (MPA60, MPA223, and MPA645) = "*Candidatus* M. parvicella", **Myc657** = nocardioform actinomycetes, **DIE993** = *Dietzia* spp., **R.ery619** = *Rhodococcus erythropolis*. **Hydrophobicity** values in % (**MATH**, mean value and error bar = standard deviation calculated from 5 replicates) determined for all samples before and after EPS extraction.

The hydrophobic properties (MATH) showed an increase for all scum samples as compared to activated sludge no matter if nocardioform actinomycetes or *M. parvicella* were present in high numbers (Fig. 30). After EPS extraction for all samples examined slightly higher MATH values were analyzed as compared to the non-treated samples showing floc break-up released hydrophobic sites.

The application of the MAC method showed an attachment of fluorescent hydrophobic microspheres to the cell surface of *M. parvicella* in all samples examined independent of the frequency of *M. parvicella* (Fig. 31 A and B). In contrast, no microspheres adhesion to the cell surface of the nocardioform actinomycetes was observed (Fig. 31 A).

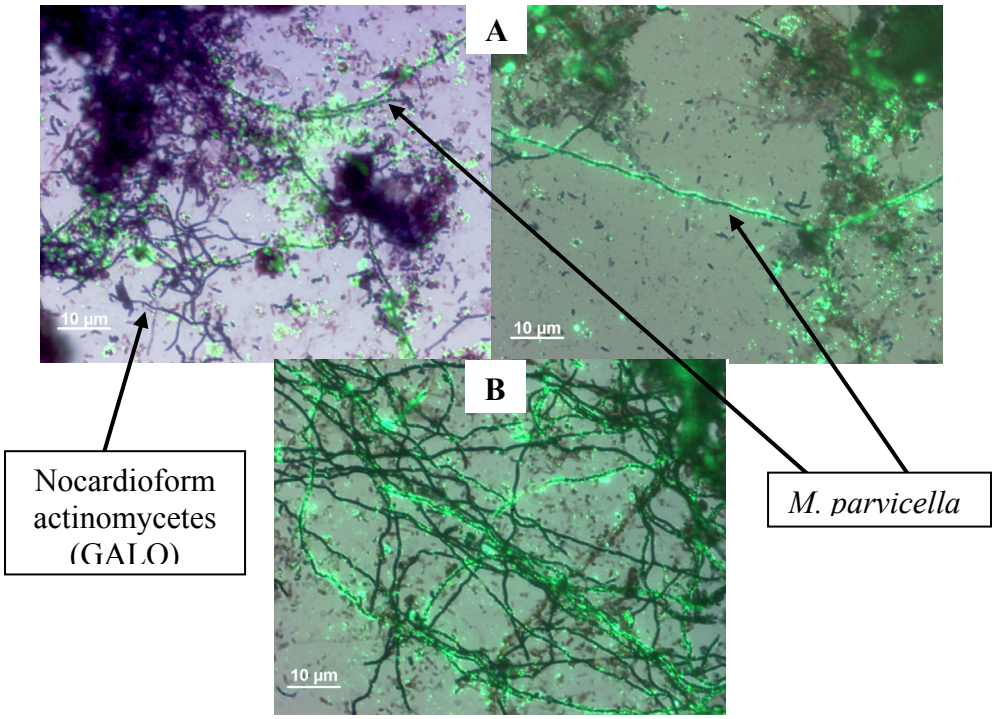


Figure 31: Bright field images showing *M. parvicella* and nocardioform actinomycetes (GALO) after Gram staining superimposed with epifluorescence images demonstrating green fluorescent hydrophobic microspheres in scum samples from the WWTP-BT collected in May 2003 (A) and July 2003 (B).

5.3.3.2 EPS characterization

An increase of the TOC content from 55 to 96 mg L^{-1} was observed in the primary effluent from May until November 2003 followed by a decline in February 2004 (Fig. 32). In the aeration tank water phase significantly lower TOC concentrations were determined (9 to 26 mg L^{-1}). The EPS fractions of activated sludge and scum yielded TOC values from 76 to 108 mg g^{-1} VS, i.e. within a similar range as was detected for WWTP-M. Only slight differences in the TOC content of the activated sludge EPS and scum EPS were analyzed besides November 2003. For these samples a significantly higher TOC content was determined for the scum EPS as compared to the corresponding activated sludge EPS.

Protein and carbohydrate concentrations calculated in TOC units are shown in Figure 32. The primary effluent contained protein at a concentration of 8 to 32 mg L^{-1} and a significantly lower concentration of carbohydrate (protein to carbohydrate ratio of 4 to 5). In contrast, both protein and carbohydrate were detected in similarly low amounts in the aeration tank water phase (2 to 6 mg L^{-1}, protein to carbohydrate ratio of 1 to 2). A protein content of 24 to 43 mg g^{-1} VS was determined for the EPS of activated sludge and scum with a usually slightly higher concentration in the scum EPS. In July 2003 the lowest protein amount within the EPS was detected for both activated sludge and scum with a slightly lower protein content in scum EPS. For all EPS samples a lower content of carbohydrate was analyzed and a protein to carbohydrate ratio of 4 to 6 was calculated.

Very low amounts of lipophilic substances enter the system by the primary effluent (< 1 to 3 mg L^{-1}), only in February 2004 a higher amount of 10 mg L^{-1} was detected (Fig. 32). In both

aeration tank water phase and extracted EPS very low amounts of lipophilic substances were determined over the entire sampling period (water phase < 1 to 2 mg L^{-1}; EPS 1 to 4 mg g^{-1} VS). A high fraction of residual TOC representing 60 to 86% of the TOC in the primary effluent and 36 to 62% of the TOC in the EPS remains to be analyzed in detail.

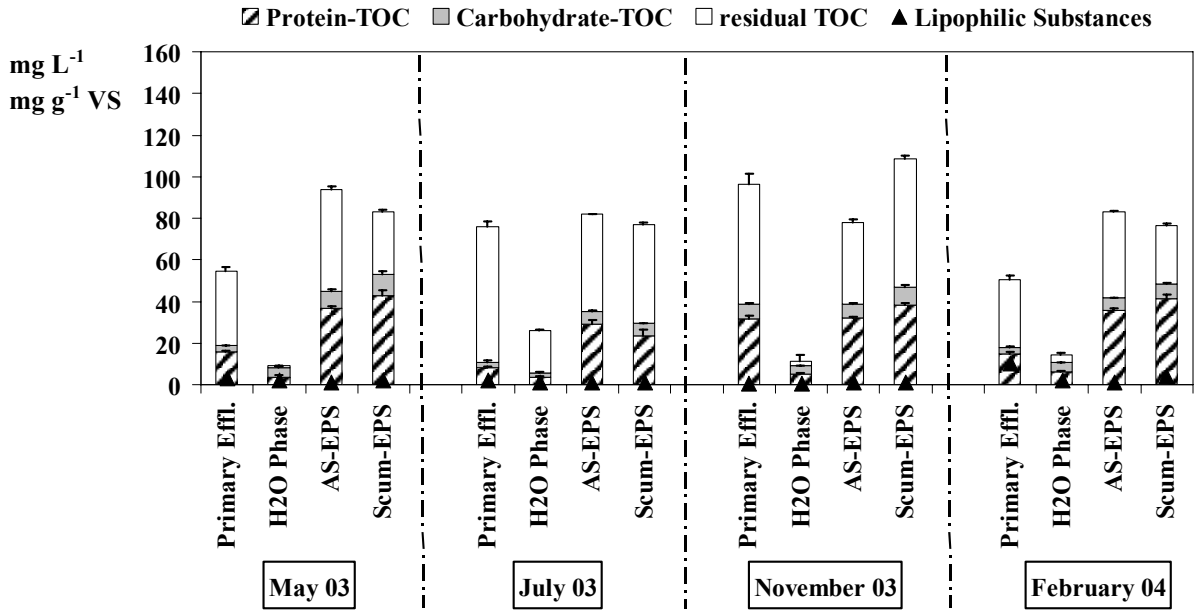


Figure 32: TOC (mean value and error bar = standard deviation from 2 replicates), carbohydrate and protein content (determined as TOC units, mean value, and error bar = standard deviation calculated from 3 replicates), and lipophilic substances in the primary effluent [mg L^{-1}], aeration tank water phase [mg L^{-1}], and EPS [mg g^{-1} VS] extracted from activated sludge (AS) and scum of WWTP-BT.

5.3.4 Scum Bacteria and Respiratory Activity

Viability and respiratory activity of the microbial biocenosis from both activated sludge and scum were investigated for the three WWTPs described above. This approach might give information whether the dominant organisms present in the scum fraction passively floated to the surface or else active growth took place in the scum layer. The reduction of CTC by the respiratory electron transport chain to insoluble, fluorescent formazan crystals (CTF) determines the respiratory activity and viability of bacteria. In this study the formazan deposits were determined both in vitro by spectrophotometric analysis after formazan extraction with ethanol and in situ by CLSM. In addition, the dominant scum bacteria were quantified in activated sludge and scum by FISH.

The respiratory activity measured by spectrophotometry and the frequency of the dominant scum bacteria are shown in Figure 33. In WWTP-M a high activity was determined in activated sludge from AT1 and distinctly lower activities were detected in activated sludge from AT2. This result is plausible because of the higher substrate supply in AT1 (high load stage) in comparison to AT2 (low load stage). Furthermore significantly higher CTF values were analyzed for the scum samples from AT2 and AT1 as compared to the corresponding activated sludge. This observation correlated with high numbers of nocardioform

actinomycetes in the scum fraction (26 to 53% of the total bacteria), which were mostly identified as *Rhodococcus* sp. cluster B accompanied by some *Dietzia* spp..

In contrast, a decline of the respiratory activity was determined for scum as compared to activated sludge in WWTP-GR. In both activated sludge and scum "*Candidatus* M. parvicella" was detected quite frequently (10 to 35% of the total bacteria) with a clear enrichment in the scum fraction despite a lower respiratory activity.

A variation of respiratory activity was observed for the sludge samples originating from WWTP-BT on different sampling days characterized by a shift of the dominant bacterial biocenosis. The highest increase of the produced CTF in the scum layer as compared to activated sludge was detected in May 2003, corresponding with the highest numbers of nocardioform actinomycetes in scum (38% of the total bacteria) identified as *Rhodococcus erythropolis* and *Dietzia* spp.. In July 2003, when "*Candidatus* M. parvicella" was found frequently in activated sludge and scum (26% and 48% of the total bacteria, respectively), the opposite was observed. No differences in the activity of both activated sludge and scum were determined in November 2003. On this sampling date nocardioform actinomycetes were clearly enriched in the scum fraction but occurred in quite lower numbers in comparison to May 2003. An increase of nocardioform actinomycetes mostly represented by *Rhodococcus erythropolis* from November 2003 to February 2004 was observed in the scum fraction and simultaneously a higher respiratory activity was detected in the scum as compared to the corresponding activated sludge.

In situ determination of CTF-positive cells was carried out after DAPI staining using CLSM. The dominant scum bacteria nocardioform actinomycetes and *M. parvicella* were identified by their different conspicuous morphologies. It was shown that nocardioform actinomycetes detected in scum from WWTP-M (Fig. 34 A) and WWTP-BT (Fig. 34 B arrowed, May 2003) were quite active as were *M. parvicella* in activated sludge from WWTP-GR (Fig. 34 C) and WWTP-BT (Fig. 34 E, July 2003). However, not all cells of these scum bacteria showed a positive CTC reduction as was observed for other bacteria as well. In contrast to the activated sludge, *M. parvicella* filaments detected in scum produced a lower amount of formazan crystals (Fig. 34 D and F) confirming *M. parvicella* might be less active in scum as compared to activated sludge.

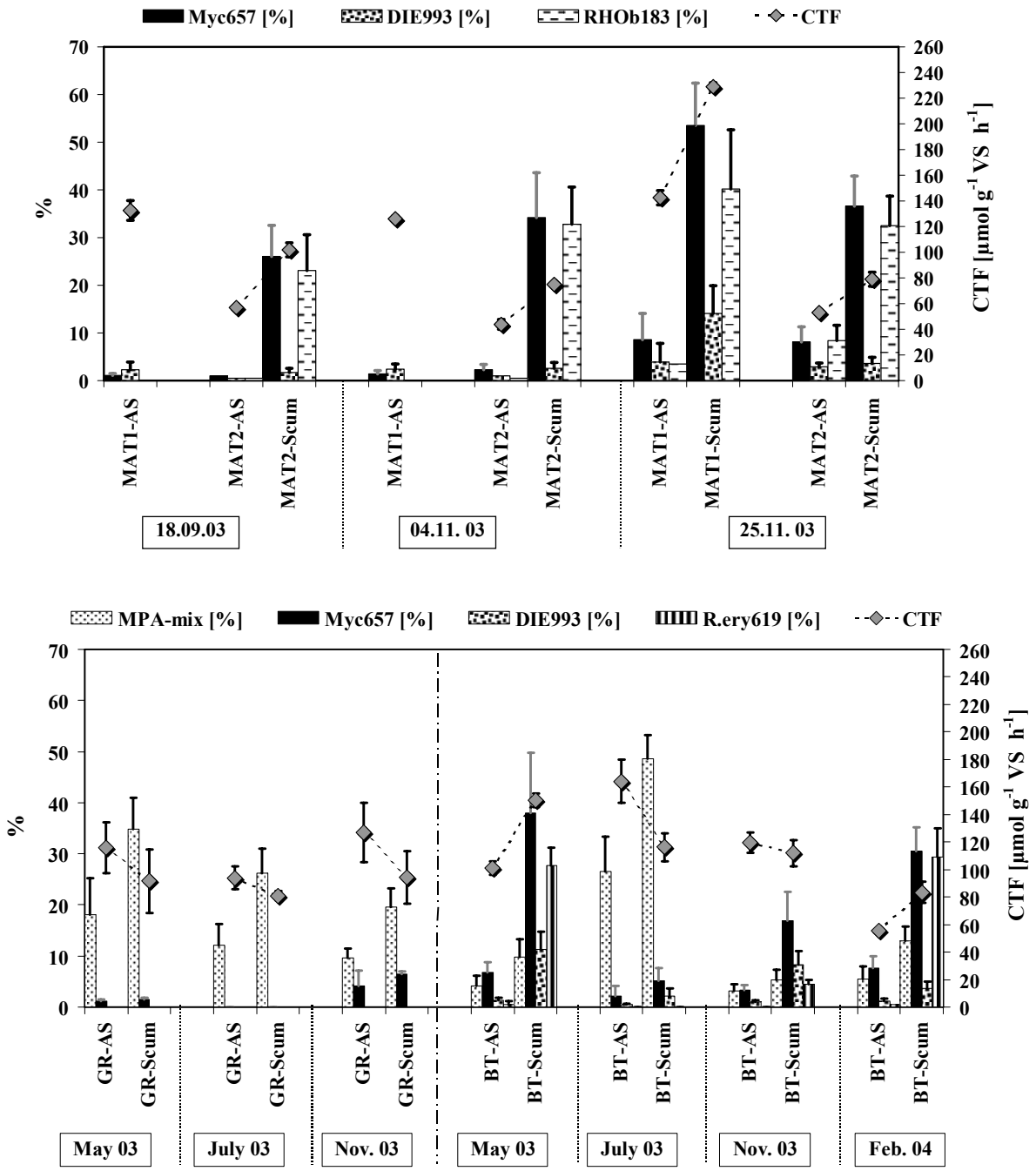


Figure 33: Respiratory activity measured spectrophotometrically as formazan (CTF, mean values and error bar = standard deviation calculated from 3 replicates) after extraction with ethanol in activated sludge and scum originating from different WWTPs (MAT1, MAT2, GR, and BT). In addition, the dominant scum bacteria were detected and quantified by FISH with nocardioform actinomycetes-specific probe Myc657, *Dietzia*-specific probe DIE993, *Rhodococcus*-specific probes RHOb183 = *Rhodococcus* sp. cluster B and R.ery619 = *Rhodococcus erythropolis*, and "*Candidatus* M. parvicella"-specific probes MPA-mix = MPA60, MPA223, and MPA645. Percentage area fractions of a specific probe related to EUB338-mix probes detecting the domain *Bacteria* were calculated (mean value of 15 to 20 images, error bar = corresponding standard deviation).

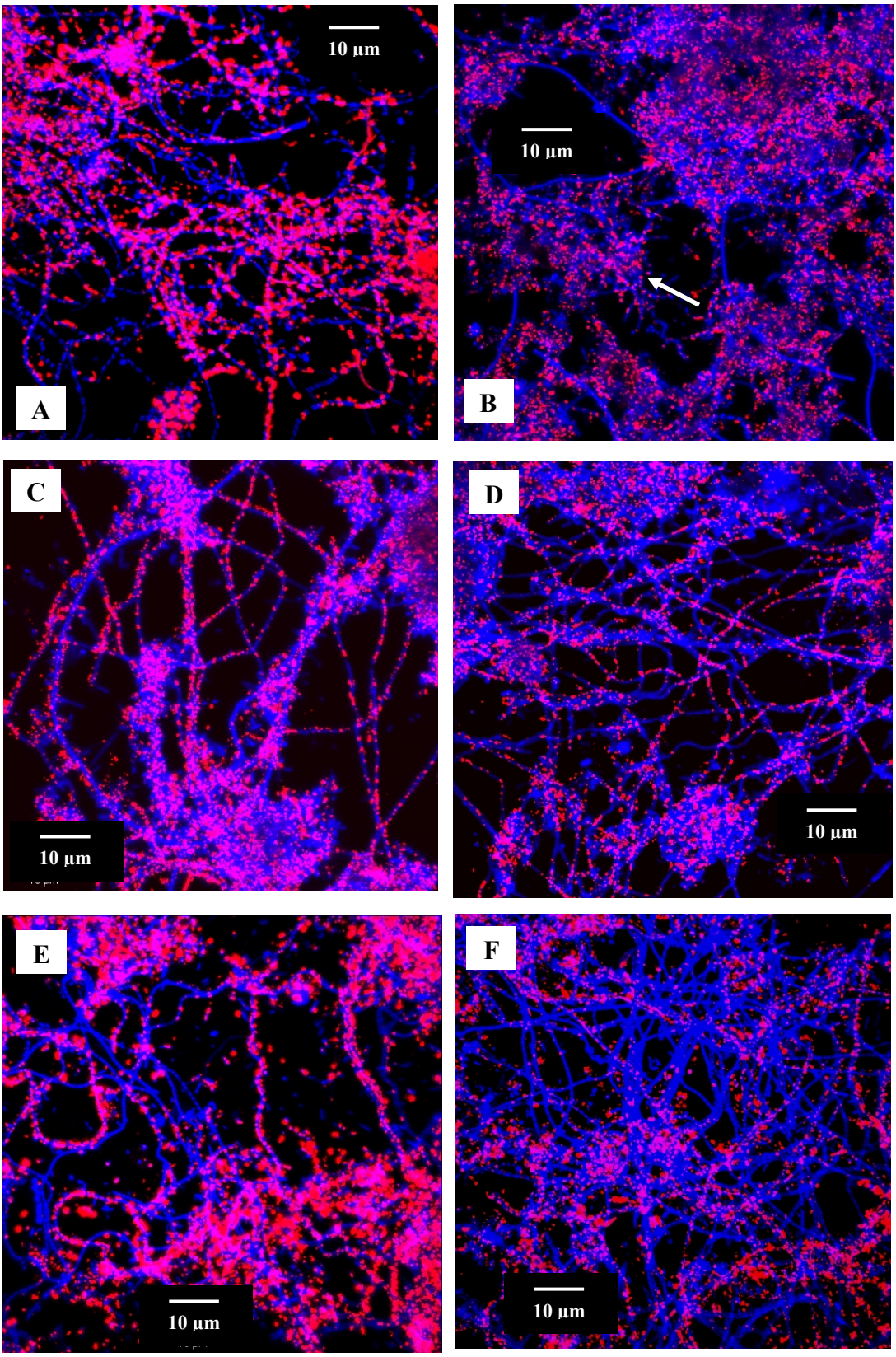


Figure 34: CTC (red) and DAPI (blue) staining of the dominant scum bacteria in scum and activated sludge. CLSM images showing branched nocardioform actinomycetes detected in scum from WWTP-MAT2 (A), *M. parvicella* found in activated sludge (B) and scum (C) from WWTP-GR, branched nocardioform actinomycetes detected in scum collected in May 2003 (D) and *M. parvicella* detected in scum collected in July 2003 (E) from WWTP-BT. The overlaps between the CTC and DAPI signals are shown as magenta.

6 DISCUSSION

6.1 Possibilities and Limitations of Classical Microscopic Sludge Analysis and FISH to Characterize Scum Bacteria

The classical approach to identify filamentous scum bacteria based on morphological features and different staining properties provides reliable detection results on the level of morphotypes shown by many survey studies all over the world (see chapter 2.3.2, Table 1). However, the question arises whether the morphotypes detected are to be classified as one group, genus or species phylogenetically. Behind one morphotype might be concealed different taxonomic organisms with various physiological properties, which consequently require different control strategies. In addition, it is known that morphological criteria and classical staining techniques using Gram and Neisser dyes vary greatly at different environmental conditions and are consequently not always effective tools to characterize these organisms. For *M. parvicella*, nocardioform actinomycetes, and many others non-filamentous growth forms are documented, which are not detectable by classical sludge microscopy (Foot et al. 1992, Müller et al. 2002, Hug et al. 2005). Furthermore morphological features, e.g. sheath forming, might be plasmid encoded and thus easily lost (Emerson and Ghiorse 1992). Hence additional identification tools, e.g. fluorescence in situ hybridization (FISH) using fluorescently labeled rRNA-targeted oligonucleotides, are required to get more detailed information at various taxonomic levels. Different work groups developed several oligonucleotide probes specific for filamentous bacteria involved in scum process during the last years (for details see chapter 2.3.2 and 4.2.2.2, Table 2). One aim of this study was to compare and evaluate the classical microscopic sludge analysis and FISH for detecting and quantifying scum bacteria. In the following (i) possibilities and limitations of both methods for each morphotype are discussed and (ii) a general evaluation is given.

Morphotype *M. parvicella* was proved after application of FISH using several specific oligonucleotide probes (Erhart et al. 1997) to be identified in all samples as "*Candidatus* Microthrix parvicella", an unclassified member of the Actinobacteria (Blackall et al. 1994b, Blackall et al. 1996b). This observation agreed with the findings by Erhart et al. (1997), who suggested this very characteristic morphotype to be one single phylogenetic entity found in different WWTPs of Germany, France, and Australia. Hence classical microscopic sludge analysis seems to be a successful tool to identify this organism and would only fail if *M. parvicella* showed a variation in its morphology or Gram stain reaction. This was observed by Foot et al. (1992) after feeding experiments on a selective substrate (Tween 80) and by Paris et al. (2005) after aluminum dosage. At these conditions the FISH technique proves to be the more reliable identification tool. However, the detection of *M. parvicella* by the FISH method revealed also some problems. *M. parvicella* filaments in this study were not visualized with the *Actinobacteria*-specific probe HGC69, despite "*Candidatus* M. parvicella" is an unclassified member of the *Actinobacteria* phylum. This was confirmed by Erhart et al. (1997). Bradford et al. (1998) showed that the 23S rRNA of "*Candidatus* M. parvicella" has three mismatches to the *Actinobacteria*-specific probe HGC69a, preventing probe binding. In contrast to the HGC69a probe, the application of the probe HGC1156 targeting a 16S rRNA region specific for the phylum *Actinobacteria* (Erhart 1997) results in positive FISH signals for *M. parvicella*. These observations demonstrate that the probes which are going to be

applied have to be carefully chosen and it is very important to check the probe sequence against the target sequence using specific probe match software tools. Furthermore to obtain optimal fluorescent signals for *M. parvicella*, the standard FISH protocol had to be optimized. Based on the results of this study it is recommended to use enzymatic pre-treatment (lysozyme), which has been advised for the complex cell wall of the Gram-positive bacteria (Beimfohr et al. 1993). However, the detection of the whole *M. parvicella* filament was successful only after extending the hybridization time to at least 15 h. A lower hybridization time of 2 h resulted in a fragmental visualization of the filament only, which made it difficult to estimate the real amount of *M. parvicella* and also made identification difficult for less experienced staff.

The Gram-positive branched filamentous morphotypes (GALO, *Gordonia amarae*-like organisms) were easily identified as **nocardioform actinomycetes** (NOC) by classical microscopic techniques. However, the method is not reliable to classify Gram-positive bent rods growing as short non-branched filaments or cell clusters (NOC-like organisms). Only the application of FISH using specific probes for nocardioform actinomycetes, MNP1 (Schuppler et al. 1998) and Myc657 (Davenport et al. 2000), detected both GALO and NOC-like organisms and showed them to belong to the taxonomic nocardioform actinomycete group. Also single rods not detectable by classical microscopy were only visible and identified as nocardioform actinomycetes by the FISH method. Nocardioform actinomycetes should be detected and identified in all their different growth forms from branched filaments to coryneform single cells. As both their hydrophobic cell surfaces and their ability to produce biosurfactans (Bendinger et al. 1993) are reported to strongly support scum formation and stabilization (Blackall and Marshall 1989). The higher detection rate of probe Myc657 as compared to probe MNP1 is explainable by probe specificity examined by different probe match software tools (BLAST, Altschul et al. 1990, RDP II, Cole et al. 2003, ARB software package, Ludwig et al. 2004) and the hybridization conditions used. It was shown that the 16S rRNA sequence of some *Mycobacterium* spp., *Corynebacterium* spp., *Rhodococcus* spp., *Tsukamurella paurometabola*, and *Skermania piniformis* had one mismatch with probe MNP1. Furthermore some species of other genera within the *Actinobacteria* (e.g. *Arthrobacter*, *Micrococcus*, *Brevibacterium*, *Kocuria*, *Streptosporangium*, and *Actinomadura*) are targeted by probe MNP1. In contrast to these observations, probe Myc657 detected only members of the nocardioform actinomycete group. However, this probe also does not match the sequences of all species within this group. The probe exhibited one mismatch with several members of the genus *Nocardia*, two mismatches with some *Rhodococcus* spp. (*Rhodococcus* cluster A and B, detected in this study and by Soddell et al. 1998), and mostly three mismatches with most *Corynebacterium* spp. and *Rhodococcus fascians*. However, Davenport et al. (2000) determined hybridization conditions with a stringency targeting organisms including nocardioform actinomycetes whose sequences exhibited one mismatch with the probe sequence. The sequences of non-target organisms exhibited two or more mismatches with the probe sequence and are consequently not binding to the probe under the stringency conditions recommended for hybridization. It was shown that probe Myc657 is a more reliable probe than probe MNP1 for the identification of nocardioform actinomycetes involved in scum formation which were represented by *Gordonia* spp., *Rhodococcus* spp., *Skermania piniformis*, *Dietzia* spp., *Tsukamurella* spp., and *Mycobacterium* spp. (see chapter 2.3.2.2, Goodfellow et al. 1996, Soddell et al. 1998, Seong

et al. 1999, Stainsby et al. 2002, Nam et al. 2003). Classical microscopy is not able to distinguish between these various genera, which in general grow as right-angled filaments. In addition, non-branched filamentous and non-filamentous growth is described in literature (Schuppler et al. 1998, Davenport et al. 2000, Müller et al. 2002, Hug et al. 2005) and was as well found in the present study for nocardioform actinomycete species. Only *Skermania piniformis*, which develops a distinctly different branching at acute angles, can easily be identified on the basis of its morphology. The application of FISH using probes for *Gordonia* spp. and *Skermania piniformis* failed to identify the nocardioform actinomycete group at lower taxa levels in German WWTPs. Only the development of new probes carried out in this study demonstrated that *Dietzia* spp. characterized as NOC-like organisms and different *Rhodococcus* spp. growing as typical right-angled branched filaments were present in high numbers. Identification at group level is not sufficient for the nocardioform actinomycetes associated with scum because different species are known to be characterized by specific physiological properties (see chapter 2.3.2.2, 6.2.2, and 6.3), which might influence the scum process differently and demand selective control strategies. Therefore detection at genus or species level is required, which is only possible by the FISH method. However, at this point the deficit of genus- and species-specific probes turned out to be one limitation of FISH. Insufficient cell wall permeability represents an additional problem during detection and identification of some nocardioform actinomycete species by FISH. Even after applying different pre-treatments recommended in the literature, e.g. enzymatic treatments and mild acid hydrolysis (Beimfohr et al. 1993, Davenport et al. 2000, Carr et al. 2005), and increasing the hybridization time to 15 h, the filaments of some species were only partly detectable or else FISH signal intensity was low, e.g. after mild acid hydrolysis. Moreover a low ribosome content providing not enough rRNA target molecules might be responsible for these detection troubles. Eales et al. (2005) discussed PTLO detected in foam probably suffer starvation leading to lowered cell rRNA contents for single cells in the filament. Hence fewer target sites for the FISH probes are available resulting in uneven regions of strong fluorescence and no fluorescence at all.

Filamentous bacterial morphotypes denoted by numbers such as **1863, 0041/0675, 1851, and 0092** were found generally at low numbers. Nevertheless some information about their taxonomic position was revealed. **Type 1863** is described to be not one of the major scum-causing filaments in activated sludge systems (see Table 1, chapter 2.3.2). However, in the present study this filamentous organism was found in some plants enriched in the scum fraction. Wagner et al. (1994) found the oligonucleotide probe specific for *Acinetobacter* spp. to bind to activated sludge filaments that had been identified by morphological features as type 1863. This observation corresponds very well with the results of the present study where the filamentous bacteria type 1863 and in addition coccoid cells were characterized as *Acinetobacter* spp. by FISH. In the literature type 1863 is described to belong to various taxonomic groups. Rossetti et al. (1997b) isolated type 1863 from an Italian WWTP and identified this isolate on the basis of phenotypic characteristics and 16S rDNA sequence data as *Acinetobacter johnsonii*. Five type 1863 isolates obtained from Australian plants showed taxonomic diversity (Blackall et al. 1996a, Seviour et al. 1997). Two isolates belonged to the genus *Acinetobacter* and one was a *Moraxella* sp. most closely related to *Moraxella osloensis*. In addition, two isolates were affiliated with the *Chryseobacterium* subgroup (formerly the *Flavobacterium* subgroup) within the *Bacteroidetes* phylum. These data demonstrate that type

1863 found in different countries does not represent one single genetic entity. At least three different organisms classified into various bacteria groups are able to develop similar morphological features.

The phylogenetic affiliation of **type 0041/0675** on the basis of 16S rDNA analysis has not yet been carried out. However, Hugenholtz et al. (2001) demonstrated that the application of FISH using specific probes for the TM7 division (TM7905 and TM7305) visualized a conspicuous Gram-positive sheathed filamentous bacterium in a laboratory scale activated sludge that matched morphologically the description of morphotype 0041/0657. The TM7 division is a candidate division of the domain *Bacteria* currently known only from environmental 16S rDNA sequence data (Hugenholtz et al. 1998). This division represents the third major lineage of the domain *Bacteria* that includes members with Gram-positive cell wall features (together with the *Actinobacteria* and *Firmicutes*). Most types 0041/0675 found in the present screening study gave good signals with probe TM7905. A lower number of 0041/0675 filaments was detected by probe TM7305, being usually applied simultaneously with TM7905 probe. However, probe TM7905 highlighted filaments that were characterized by different morphological features. These observations were confirmed by Hugenholtz et al. (2001), who detected type 0041 filaments by probe TM7305 in different sludge samples originating from a laboratory scale reactor and two Australian WWTPs. In addition, they showed that probe TM7905 binds to TM7305-positive filaments, thick filaments, and single coccoid cells. Similar results were reported by Thomsen et al. (2002) investigating type 0041 in two Danish WWTPs. This is consistent with the broader phylogenetic specificity of probe TM7905 targeting nearly the entire TM7 division whereas probe TM7305 targets most of the members of the TM7 subdivision 1 only. Furthermore in the present study it was shown that not all 0041/0675 morphotypes detected by classical microscopy belonged to the TM7 division. Thomsen et al. (2002) also reported that only a part of the detected morphotype 0041 showed positive FISH signals applying TM7 probes. All other filaments morphologically identified as type 0041 hybridized with the EUB338 probe only. No signals were observed after the application of other group-specific bacterial probes. This observation indicates that the TM7-negative type 0041 filaments probably do not belong to the presently established bacterial phyla, e.g. *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. It is quite likely that type 0041/0675 represents a diverse organism group of which TM7-positive filaments are only one example. However, a distinct increase in TM7 filament numbers in sludge during a foaming episode is reported by Hugenholtz et al. (2001) indicating that filamentous bacteria belonging to the TM7 division might play a role in scum development. These TM7-positive filaments might comprise more than one species or even genus of the TM7 division, since the probes target a broad phylogenetic group and morphology is a poor indicator of phylogenetic conformity.

The 16S rDNA sequence analysis of isolated **filamentous type 1851 bacteria** from bulking activated sludge originating from an industrial WWTP in Japan (Kohno et al. 2002) and from a WWTP in Australia (Beer et al. 2002) revealed that all these isolates, independently of their origin, represent an unclassified member of the *Chloroflexi* phylum (subdivision 3, Björnsson et al. 2002) closely related to *Roseiflexus castenholzii* but only with 84% sequence similarity. The application of probe CHL 1851 (Beer et al. 2002) designed against the sequence of the isolated type 1851 showed that all morphologically characterized types 1851 in activated sludge gave positive FISH signals in the present study. Moreover Beer et al. (2002) detected

morphotype 1851 in samples collected from plants in different countries, e.g. Australia, New Zealand, France, and the Netherlands, that responded to the CHL 1851 probe. On the basis of these results type 1851 is probably phylogenetically very similar regardless of its origin.

The identification of **morphotype 0092** by FISH was not successful at all. Bradford et al. (1996, 1997) analyzed the near complete 16S rDNA sequence of an isolated filamentous type 0092 and showed that this isolate belonged to the *Flavobacterium* subgroup (formerly *Cytophaga*), most closely related to *Flavobacterium columnare* within the *Bacteroidetes* phylum. None of the detected type 0092 filaments in the present study gave a positive FISH signal with the specific probe 0092-997 (Bradford 1997) targeting the 16S rRNA sequence of the type 0092 isolate described above. The characterization of type 0092 requires further phylogenetic research to obtain taxonomic information on this morphotype.

Eikelboom and van Buijsen (1983) described three morphotypes of ***N. limicola***, i.e. I, II, and III, differentiated mainly by their cell dimension, cell morphology, and filament shape. This observation points out either *N. limicola* to be a single bacterium existing as several morphological variants or *N. limicola* morphotypes to consist of different organisms. It was shown on the basis of 16S rDNA sequence analyses of several isolates that the second assumption is correct (Blackall et al. 2000, Liu et al. 2000, Liu et al. 2001, Schade et al. 2002, Snaidr et al. 2002). The three morphotypes of *N. limicola* make up members of at least five different phylogenetic lineages. ***N. limicola* I** consists of at least two bacterial genera, *Trichococcus* and *Streptococcus*, within the *Firmicutes* phylum (Liu et al. 2000). Liu and Seviour (2001) showed that probe NLIMI 91 targeting *Trichococcus* species only detected short filaments and single coccoid cells in different activated sludges originating from Australia, Italy, and France. However, the probe failed to identify the typical *N. limicola* I morphotype staining Gram-positively and appearing as long chains of cocci. Similar results were obtained in the present study. This clearly demonstrates that the filamentous morphotype *N. limicola* I implies more than one single bacterial entity. ***N. limicola* II** is described as the most diverse morphotype within the *N. limicola* group. Blackall et al. (2000) characterized *N. limicola* II on the basis of 16S rDNA sequence and chemotaxonomic data to be a novel genus within the family *Intrasporangiaceae* (*Actinobacteria* phylum) presently referred to as "*Candidatus* Nostocoida limicola". The application of specific probes (Bradford 1997, Liu and Seviour 2001) for this organism demonstrated "*Candidatus* N. limicola" to be found quite frequently in different activated sludge samples as was shown for WWTPs in Australia, New Zealand, and France (Liu and Seviour 2001) and for WWTPs of the present study. Other *N. limicola*-like organisms clustered within the *Chloroflexi* phylum with *Sphaerobacter thermophilus* as the nearest published relative (Schade et al. 2002) or in the class of *Alphaproteobacteria* classified into novel taxa (Snaidr et al. 2002, Levantesi et al. 2004). They were, however, detected only in low numbers in the sludge samples of the present screening investigations. Liu et al. (2001) described ***N. limicola*** III isolates to be affiliated with the *Planctomycetes* phylum, closely related to *Isosphaera pallida*. The authors (Liu and Seviour 2001) claim probe NLIMIII 301 to target both isolated *N. limicola* III strains and *Isosphaera pallida*. However, in the present study none of the *N. limicola* III morphotypes was visualized by this specific probe. On the contrary only *Isosphaera* morphotypes identified according to Staley et al. (1992) could be detected with this probe. The slightly bent *Isosphaera* filaments are characterized by coccoid cells with a characteristic transparency presumably caused by gas vacuoles. In contrast, the *N. limicola* III morphotype originally

described by Eikelboom (1975, Eikelboom and van Buijsen 1983) develops strongly twisted filaments consisting of coccoid to disc-shaped opaque cells, which can be easily distinguished from the *Isosphaera* morphotype. Consequently this organism is assumed to belong to a different taxonomic group. The findings described and the various literature data emphasize the advantage of using FISH to identify *N. limicola* filaments as this morphotype contains several phylogenetically unrelated bacteria. However, for all three *N. limicola* morphotypes, the available probes are still insufficient with a particular deficit on taxonomic knowledge about the morphotypes *N. limicola* I and III.

The application of both classical microscopic sludge analysis and FISH demonstrated that most filamentous morphotypes described are characterized by various taxonomic groups, genera, and species, which possess specific physiological properties (for details see chapter 2.3.2) and consequently demand various control measures. In contrast, the characteristic morphotype *M. parvicella* turned out to be one single phylogenetic entity. Therefore a selective control strategy against *M. parvicella*, e.g. aluminum dosage, might be effective all over the world, which is verified by various reports (Eikelboom et al. 1998, Lind and Lemmer 1998, Nielsen et al. 2005, Paris 2005). Classical sludge analysis turned out to be a successful tool to identify this organism. Similar results were obtained for branched filamentous nocardioform actinomycetes, type 1863, and type 1851 in the present study. The microscopic sludge analysis failed to detect the non-filamentous morphotypes of some organisms as was demonstrated for nocardioform actinomycetes and type 1863. These non-filamentous bacteria could be classified only after applying FISH as the more sensitive detection method. In addition, only the FISH technique disclosed the phylogenetic diversity within the described morphotypes 0041/0675, 0092, and *N. limicola*. However, the limitation of FISH to identify these morphotypes was also revealed. There is still a deficit of adequate probes to describe these morphotypes at the phylogenetic level, subsequently causing a lower detection frequency as compared to classical microscopy. Furthermore available probes give only information at higher taxa level. Further information at the genus and species level is required, e.g. for the highly diverse nocardioform actinomycete group. Moreover the FISH method has to be optimized for the various organisms with respect to pre-treatment and probe detection specificity.

6.2 Nocardioform Actinomycetes (*Corynebacterineae*)

6.2.1 Molecular biological Methods – Identification of Nocardioform Actinomycete Species

Various work groups demonstrated that less than 15% of the total bacteria in activated sludge were cultivable, and in addition culture-dependent community analysis of activated sludge produced partial and heavily biased results (Wagner et al. 1993, Kämpfer et al. 1996). Selective isolation and identification of branched filamentous nocardioform actinomycetes using micromanipulation followed by sequencing and sequence analysis were successfully performed for *Rhodococcus* spp. (Soddell et al. 1998), *Gordonia amarae*, and *Skermania piniformis* (Blackall et al. 1994a). However, this method would fail, e.g. to identify short non-branched filamentous and non-filamentous nocardioform actinomycetes, which were detected in the present investigations. Therefore in this study different culture-independent molecular biological methods, e.g. (i) the full-cycle rRNA approach including cloning, RFLP grouping,

sequencing, sequence analysis, and probe design, followed by FISH, and (ii) DGGE were used to determine the diversity of nocardioform actinomycetes involved in scum formation. The goal of the combined application of these various methods was to reduce the known limitations of each method and consequently to obtain reliable identification results (see chapter 2.4.2.5).

The cloning approach required the sequence analysis of a high number of clones to obtain adequate information about the different operational taxonomic units within clone libraries and consequently of the habitat examined. This is very time-consuming, labor-intensive, and expensive. Therefore the RFLP technique was used to profile different clone libraries into taxonomic groups. This method is described to be extremely useful for grouping of clones originating from samples that are characterized by low microbial diversity (Bond et al. 1995, Burrell et al. 1998). This might be the case for all scum samples analyzed, which were enriched with high numbers of nocardioform actinomycetes. However, the RFLP method failed to group the clones from the TH scum library resulting in high numbers of RFLP groups composed mostly of individual clones indicating that the diversity of the community is too high as to be simplified by RFLP. Sequencing of different clones followed by sequence analysis confirmed the high diversity within this clone library. The dominant group within the scum biocenosis identified by FISH as nocardioform actinomycetes was represented only by few clones that were all classified as *Skermania piniformis*. In contrast, in situ analysis applying FISH using *Gordonia*- and *Skermania piniformis*-specific probes frequently detected both organisms within the scum samples and demonstrated the limitations of the cloning technique to reflect the microbial community composition of the original samples. Although the PCR cloning approach has been successfully applied to characterize different microbial habitats (Borneman and Triplett 1997, Snaidr et al. 1997, Dojka et al. 1998) it should be stressed that this molecular genetic approach is not completely unbiased. Several factors such as DNA extraction from samples, PCR amplification bias, gene copy number bias, and cloning and sequencing artefacts might provide incorrect data (for details see chapter 2.4.2.2.). These data do not reflect the true diversity of the original sample as was shown for the clone library TH in this study. It should also be noted that the analysis was probably not comprehensive enough and the selected clones represented only a small number of the various sequences within the clone library. These results demonstrated that it is very important to control the sequencing results obtained from clone libraries with further molecular biological techniques, e.g. FISH or DGGE, to reveal the diversity within the original samples.

Because of the low success of the RFLP method described above and the focus on the taxonomic description of the nocardioform actinomycete group within the phylum *Actinobacteria* the clone libraries MAT1, MAT2, and BT (German WWTPs) were screened with *Actinobacteria*-specific primers before applying RFLP to reduce the microbial diversity. The RFLP profiling of *Actinobacteria*-positive clones was proven extremely useful in grouping clones in the libraries MAT1, MAT2, and BT. Partial sequence analysis demonstrated that RFLP groups in general represent operational taxonomic units. Furthermore for all three clone libraries, MAT1, MAT2, and BT, the dominant RFLP group represented species within the nocardioform actinomycete group. This confirms that the diversity within the clone libraries described the dominant taxonomic group within the *Actinobacteria* phylum of the scum biocenosis detected by FISH. However, because clone libraries are not considered to reflect the true microbial composition, the results at species

level needed to be proved by further molecular biological techniques to show whether the nocardioform actinomycete species, *Dietzia* spp., *Gordonia* sp., *Mycobacterium* sp., and different *Rhodococcus* spp. detected within the clone libraries represent the diversity of the nocardioform actinomycetes within the original microbial community.

The DGGE technique has been performed to determine the redundancy in clone libraries and to estimate the abundance of particular cloned 16S rDNA inserts in natural habitats (Kowalchuk et al. 1997). In the present study DGGE was used in combination with RFLP, which was a reliable tool to estimate the occurrence of dominant clone inserts detected by RFLP in the microbial community analyzed. This strategy represented an advantage as compared to the application of each technique alone. RFLP is less labor-intensive for screening a high number of clones as compared to DGGE, which is necessary to obtain an overview about the diversity within a clone library as was discussed above. However, only DGGE can compare the diversity within the clone library and the original sample, and represents consequently the more successful tool to select clone inserts for further analyses including full length sequencing, sequence analysis, and probe design followed by FISH. The prerequisite of the successful application of DGGE profiling in this study was based on the screening with *Actinobacteria*-specific primers. This screening approach reduced the diversity within environmental DNA and consequently improved the DNA fragment separation of the environmental DNA by DGGE. It is known that highly diverse communities showed a complex DGGE banding pattern, which can be reduced by the analysis of PCR products with group-specific primers increasing the sensitivity of detection (Brinkhoff and Muyzer 1997, Muyzer and Smalla 1998). Heuer et al. (1997) studied the genetic diversity of *Actinobacteria* in different soils and demonstrated that the template DNA of *Actinobacteria* populations were outcompeted in the amplification process using bacterial primers and only the application of *Actinobacteria*-specific primers resulted in distinct DGGE patterns representing the *Actinobacteria* diversity of the soil samples analyzed.

The question arises whether DGGE analysis of environmental DNA followed by sequence analysis of the bands of interest might be sufficient to identify the nocardioform actinomycetes at genus and species level and consequently cloning might be unnecessary. However, there are also some limitations of the DGGE technique. For this reason a detailed population analysis requires the full-cycle rRNA approach especially in the presence of unknown species being the case in the present study. Different studies demonstrated that it is not always possible to separate DNA fragments that have a certain amount of sequence variations (Buchholz-Cleven et al. 1997, Vallaeys et al. 1997) underestimating the true microbial diversity within the original sample. Furthermore the production of double bands by the use of degenerated primers in the PCR reaction (Kowalchuk et al. 1997) and the visualization of sequence heterogeneity caused by the presence of multiple *rr*N operons with sequence microheterogeneity (Nübel et al. 1996) overestimate the number of different bacteria within natural communities. Several studies revealed that bacterial populations can only be detected by PCR-DGGE when they make up 1% or more of the total community (Muyzer et al. 1993, Murray et al. 1996). The described problems detecting the true diversity of the natural community by DGGE required the application of FISH for in situ characterization and quantification of the microbial biocenosis to verify the DGGE results. New probes usually have to be designed for this purpose representing a further limitation of DGGE at the presence of unknown species. The separation of only relatively small fragments

of up to 500 bp (Myers et al. 1985) limits the amount of sequence information. It has been found that the comparison of partial sequences is sufficient for an approximate phylogenetic assignment of an organism to a phylogenetic group if the database contains sequences from closest relatives (Ward et al. 1990, Schmidt et al. 1991, Schuppler et al. 1995). However, Ludwig et al. (1998) stated the fact that different parts of the primary structure carry information for different phylogenetic levels, which might be important for a thorough phylogenetic analysis especially for unknown species as well as for probe design. Therefore full-length sequences are required, which can only be obtained by isolation of the specific organism or by the cloning technique.

Tree topology and 16S rDNA homology values of more than 94.9% (Ludwig et al. 1998) of the full-length sequences of clone inserts proved to affiliate nocardioform actinomycetes related clones within five genera, *Dietzia*, *Gordonia*, *Mycobacterium*, *Rhodococcus*, and *Skermania*. Stackebrandt and Goebel (1994) noted that organisms with rRNA sequence similarity values less than 97.5% most likely represent different species. Based on this statement both *Rhodococcus* species clusters A and B forming two independent lines of descent within the radiation of *Rhodococcus* might represent two new *Rhodococcus* species. However, comparative sequence analysis of rRNA sequences, which provide in general sufficient information for bacterial phylogeny as well as bacterial identification at higher taxa level (Woese 1987, Ludwig and Schleifer 1994, Ludwig et al. 1998), shows only limited resolution power at the species level (Rosselló-Mora and Amann 2001). However, Fox et al. (1992) stated that although 16S rRNA sequence analysis is not a suitable method for determining new species, it can be used for identifying strains of known species. Strains exhibiting more than 99% sequence identity could be regarded as identical. Therefore the *Rhodococcus*-related clone insert (cluster A) with a sequence similarity of more than 99.7% to *Rhodococcus* spp. isolated from scum by Soddell et al. (1998) might be identical to these species. However, whether the clearly separated *Rhodococcus* clusters A and B represent new species within the genus *Rhodococcus* is not clearly determinable on the basis of 16S rRNA data. The polyphasic classification approach (Vandamme et al. 1996, including pheno- and genotyping analyses, and rRNA gene homology studies) would be necessary to identify these organisms as new *Rhodococcus* species requiring pure cultures being not available at present. Similar results were obtained for the *Dietzia*-related clones, which might be identical to different uncultured bacteria clones (sequence similarity > 99%) originating from scum collected from a WWTP situated in South Africa (Wagner and Cloete 2002). The distinct separation of this group from the remaining *Dietzia* spp. (sequence similarity of 98%) shows that probably new species within the genus *Dietzia* were detected. In contrast, a further *Rhodococcus*-related clone might be identical to *Rhodococcus erythropolis* (sequence similarity of 99.5%). The only clone sequences related to the nocardioform actinomycete group within the clone library TH (Australian WWTP) showed a sequence similarity of more than 99% to *Skermania piniformis* indicating identical species. Other clones of interest not corresponding to the dominant RFLP pattern but being visualized by distinct DGGE bands in the environmental DNA fell within the genera *Gordonia* and *Mycobacterium.* On the basis of the sequence similarity data and tree topology the *Gordonia*-related clones might be identical to *Gordonia spumae* and the *Mycobacterium*-related clones clearly separated from the other *Mycobacterium* species (97 to 98% sequence similarity) might represent a new species.

Phylogenetic analysis of the 16S rRNA data showed limitations to identify some organisms at species level as was discussed above. However, the results provide sufficient data for probe design followed by FISH to perform in situ identification of the nocardioform actinomycetes at genus and species level. As was already discussed, only the application of specific probes against the dominant sequences detected within the clone library and by DGGE analysis verified the occurrence of these organisms in the original sample. Moreover, the FISH technique only is able to reveal quantitative detection data. For the scum samples originating from German WWTPs it was shown that the dominant clone inserts defined by RFLP and DGGE profiling and the dominant DGGE bands of the environmental DNA represent most of the organisms within the nocardioform actinomycete group (*Dietzia* spp., *Rhodococcus* spp. cluster A, and *Rhodococcus erythropolis*). Other clones characterized by distinct but weaker DGGE bands as compared to the dominant clones were in general detected by FISH at lower amounts as was shown for *Rhodococcus* sp. cluster B and *Dietzia* spp. in scum MAT2 and BT, respectively, or were not detectable by FISH as was determined for *Gordonia* sp. in scum MAT2. Therefore the cloning technique combined with RFLP and DGGE profiling of *Actinobacteria*-positive clones and DGGE analysis of environmental DNA reflected the diversity of the nocardioform actinomycete group within the original microbial biocenosis. In contrast, the cloning technique describes *Skermania piniformis* as the only nocardioform actinomycete in scum from the Australian WWTP-TH and failed to detect *Gordonia* spp. whereas FISH found both organisms frequently in the scum community. The presence of *Skermania piniformis*-related clones indicates that the partial detection of PTLO filaments using *Skermania piniformis*-specific probes might be caused by a low ribosome content, which was reported by Eales et al. (2005), or limited cell wall permeability rather than by an identification error.

Nocardioform actinomycete cells are particularly difficult to permeabilize (Hahn et al. 1992), which might be attributed to their specific complex cell wall composition and, in particular, the hydrophobic nature of mycolic acids with genera-dependent variable chain length (Macnaughton et al. 1994). Several permeabilization protocols have been described in attempts to overcome this difficulty with varying levels of success (Macnaughton et al. 1994, Schuppler et al. 1998, Davenport et al. 2000, Carr et al. 2005). It was shown in the present study and by Carr et al. (2005) that there is a negative correlation of the overall size of the mycolic acid (number of carbons) and cell wall permeability. *Dietzia* spp. with the shortest chain length (C34-C38, Rainey et al. 1995b) were visualized by strong FISH signals after a gentle lysozyme pre-treatment and a hybridization time of 1.5 h. The high variation in the carbon length of the mycolic acid determined for the genus *Rhodococcus* (C34-C64, Bell et al. 1998) is reflected in a different behavior after application of various permeabilization protocols. Whereas *Rhodococcus erythropolis* and *Rhodococcus* cluster B gave positive strong FISH signals after lysozyme pre-treatment and 15 h hybridization, *Rhodococcus* cluster A was difficult to detect by FISH even after the combination of different pre-treatments resulting in partial detection of the filaments or no FISH signals. Similar results were obtained for *Skermania piniformis*, which is characterized by mycolic acids with a chain length of C58 to C64 (Chun et al. 1997). In the present study good FISH signals were revealed for *Gordonia* spp. after the same pre-treatment (lysozyme) and hybridization conditions (15h hybridization) as were applied for *Rhodococcus erythropolis* and *Rhodococcus* cluster B. However, due to the variation of the mycolic acids chain length within the genus *Gordonia* (C48-C66, Butler

and Guthertz 2001) other *Gordonia* species react differently as was shown by Carr et al. (2005). These authors discovered that the best fluorescent signals were obtained for *Gordonia amarae* using acid hydrolysis in combination with different enzymes (mutanolysin, lysozyme, lipase and proteinase K). *Mycobacterium* spp. with the longest mycolic acid chain length (C60-C90, Minnikin et al. 1984) were described to be the most difficult nocardioform actinomycete species to be detected by FISH (Davenport et al. 2000, Carr et al. 2005).

6.2.2 The Dominant Nocardioform Actinomycete Species Detected in Scum

Recent literature data on scum bacteria populations (for details see chapter 2.3.2.2) reveal a high diversity within the nocardioform actinomycete group. Most of the members of this group might be involved in scum formation. This was confirmed the first time in this study by quantitative in situ data. The results revealed *Dietzia* spp., *Gordonia* spp., different *Rhodococcus* spp., and *Skermania piniformis* to be present in high numbers in various scum samples. Furthermore the taxonomic identification at genus and species level showed that the different morphotypes described by classical microscopy belonged to different taxonomic organisms and were not the result of pleomorphism of the same organisms influenced by environmental conditions or growth phase as observed by Lemmer (1985). It was shown that the short non-branched filaments were characteristic for *Dietzia* spp., whereas the typical branched filaments were identified as *Gordonia* spp. (GALO), *Rhodococcus* spp. (GALO), and *Skermania piniformis* (PTLO).

In the present study *Rhodococcus* spp. were detected in German WWTPs only, whereas *Gordonia* spp. and *Skermania piniformis* were only found in Australian WWTPs. This corresponds well with the temperature preference of these organisms. It is known that *Gordonia* spp. and *Skermania piniformis* find optimal growth conditions at 25 to 30°C (Soddell and Seviour 1995) and at 18 to 25°C (Blackall et al. 1989), respectively. Therefore both organisms were detected more frequently in countries characterized by a warmer climate (Soddell et al. 1992), e.g. in the Australian WWTPs examined, but also in industrial WWTPs with an average wastewater temperature of 30°C (Lemmer 1985). In contrast, *Rhodococcus* spp. cells prefer a lower temperature in the range of 8 to 25°C (Lemmer 1985) and are consequently found in regions with a moderate climate (Soddell et al. 1992). Cells of the fourth genus, *Dietzia*, were present in both German WWTPs and in one Australian WWTP. These organisms were found most frequently in one German WWTP characterized by a high sludge load. In all other German WWTPs *Dietzia* spp. appeared in a distinctly lower amount together with high numbers of *Rhodococcus* spp.. In the one Australian WWTP in which *Dietzia* spp. were detected, the return activated sludge was chlorinated to control scum formation. Chlorine reduces the growth of filamentous bacteria, e.g. the typical branched filamentous nocardioform actinomycetes, which preferably grow outside of the flocs. In contrast, the *Dietzia* spp. growing as single rods or small cell clusters inside the flocs may be protected by EPS. This might be the reason why *Dietzia* spp. cells were detected in the remaining scum layer.

Nocardioform actinomycetes are able to utilize a wide range of substrates as carbon and energy sources, including readily degradable substrate as well as poorly degradable hydrophobic substrate (for details see chapter 2.3.2.2). Soddell et al. (1998) performed a detailed growth study on different nocardioform actinomycete isolates originating from

activated sludge foam. On the basis of phylogenetic analysis, these organisms were identified as *Gordonia* spp., *Skermania piniformis*, *Rhodococcus erythropolis*, and *Rhodococcus* sp. (*Rhodococcus* cluster A) representing most of the species found in the present study. These organisms were tested for growth on a number of hydrophobic substrates as sole carbon source. It was shown that they preferred to grow on the surface of insoluble hydrophobic compounds and were detected more rarely in the water phase. This observation is explainable by the hydrophobic cell surfaces described for most members of nocardioform actinomycetes, which mediates attachment to and degradation of hydrophobic substrates. All strains analyzed were able to grow on substrates containing fatty acid esters, which are important components of wastewater, and saturated aliphatic hydrocarbons, e.g. paraffin oil. *Rhodococcus erythropolis* exhibited the best growth rates under these conditions. In contrast to other studies demonsrating several nocardioforms grow well on hexadecane (Blackall et al. 1991b, Iwahori et al. 1995), Soddell et al. (1998) showed that only *R. erythropolis* have the capability to utilize hexadecane. Iwahori et al. (1995) found that *Rhodococcus* spp. had higher growth rates in activated sludge systems at higher levels of hexadecane, while *Gordonia amarae* grew more rapidly at lower levels. The described observations indicate that the several nocardioform actinomycete species found in the present study require different growth conditions. These findings are a prerequisite for the control of nocardioform actinomycetes-dominated scum by selective measures.

6.3 The Role of Scum Organisms and EPS in Scum Development

Surface-active and hydrophobic substances were shown (i) to initiate and stabilize scum formation directly (Soddell and Seviour 1990) and (ii) to promote growth of a specific bacterial scum biocenosis that excretes biosurfactants and/or is characterized by hydrophobic cell surfaces. Surface-active molecules stabilizing the gas water interface might enter the system by the WWTPs primary effluent. However, modern synthetic surfactants are in general readily degradable and not accumulating in the sludge fraction (Schade and Lemmer 2001). Due to this fact scum initialization might rather be attributed to biosurfactants produced by various organisms in the presence of hydrophobic carbon sources or nutrient deficiency (Lang 1999). Attachment to hydrophobic carbon sources followed by substrate uptake and degradation processes is enhanced by biosurfactant production and hydrophobic cell surfaces. The capacity to produce both biosurfactants and hydrophobic cell wall components is well documented for nocardioform actinomycetes (Bendinger et al. 1993, Neu 1996, Lang and Philp 1998). *M. parvicella* is also characterized by hydrophobic surface properties; however, the cause of these features is still unknown (Nielsen et al. 2002). The described hydrophobic features for both *M. parvicella* and nocardioform actinomycetes are consistent with high numbers of these organisms in scum WWTPs found in the present study and by different work groups all over the world (for details see chapter 2.3.2, Table 1). Therefore scum stabilization seems to be dependent on hydrophobic substances and a large biomass of interface-stabilizing hydrophobic bacteria.

Organisms with Gram-negative cell walls or those exhibiting attached growth such as filamentous bacteria types 0092, 1851, and 0041/0675 are characterized in general by hydrophilic cell surface properties. Thus, they are much less effective in scum stabilization (Lemmer et al. 2005). Similar observations were obtained for *N. limicola* morphotypes-

dominated sludges despite their described Gram-positive features (Khan et al. 1991, Foot et al. 1993). This corresponds well with the low detection rate of these organisms in the present study and other publications (Blackbeard et al. 1986, Goddard and Forster 1987, Wanner and Grau 1989, Lind and Lemmer 1998). They were found quite often concomitantly in *M. parvicella*- and nocardioform actinomycetes-dominated WWTPs with scum events; however, they were never found enriched in the scum fraction. Schade and Lemmer (2006) demonstrated by in situ measurements that most of these morphotypes had strong or moderate activity of different exoenzymes. This indicates they are able to utilize high-molecular-weight substrate via hydrolysis. In contrast to *M. parvicella* and nocardioform actinomycetes, no positive reaction for lipase was detected. Thus they seem not to be restricted to long-chain fatty acids as is *M. parvicella*. Their role in the scum process is still unclear and the taxonomic diversity of these morphotypes detected in the present study makes it difficult to discuss that. A seasonal fluctuation of type 0092 with a population maximum in summer and autumn, displacing *M. parvicella*, was determined in the present study and also reported by Eikelboom et al. (1998). In contrast to *M. parvicella*, type 0092 morphotype found in these studies is not dependent on reduced nitrogen compounds. Therefore type 0092 might outcompete *M. parvicella* during the summer months when complete nitrification takes place resulting in a less stable scum formation. A further filamentous bacterium, which was enriched in the scum fraction but never found to dominate activate sludge and scum biocenoses, was type 1863, identified as *Acinetobacter* spp.. Type 1863, which is not described as a typical scum bacterium, is adapted to high load sludge conditions (Williams and Unz 1985). This explains its occurrence in the high load stage of WWTP-M. Furthermore it is known that *Acinetobacter* spp. cells are able to produce emulsan, a polymeric biosurfactant, in order to ease the utilization of hydrophobic substances as carbon sources (Lang 1999). This observation indicates *Acinetobacter* spp. to contribute to the stabilization of scum layers.

Growth strategy and ecological niche of scum organisms are scarcely reported in the literature. However, to understand scum formation, it is very important to know whether these organisms are metabolically active in activated sludge causing a passive flotation of the biomass to the water surface or else an active growth process of a specific bacterial population on the water surface takes place. *M. parvicella* filaments represent approximately 15 to 26% of the total biomass in activated sludge of scum WWTPs. In contrast, population density of nocardioform actinomycetes was very low in activated sludge (< 5% of the total biomass) and significantly higher in the scum fraction (15 to 54% of the total biomass). This indicates that these organisms prefer to grow right in this fraction. This hypothesis was supported by overall respiratory activity measurements representing metabolic activity and viability of the biocenosis. The results showed that activated sludge biocenoses dominated by *M. parvicella* are more active as compared to the corresponding scum biocenoses, whereas nocardioform actinomycetes-dominated plants showed the opposite trend (higher activity in the scum fraction). The fact that overall respiratory activity refers to all bacteria and scum bacteria only constitute approximately 15 to 54% of the total bacteria, their contribution to metabolic activity has to be corroborated by enzyme activity measurements at cell level. This was demonstrated for respiratory activity in the present study and for different exoenzyme activities, e.g. lipase, phoshatase, esterase and β-glucoronindase, by Schade and Lemmer (2005, 2006). These findings indicate a different growth strategy for *M. parvicella* and

nocardioform actinomycetes. *M. parvicella* is characterized by active growth within the activated sludge floc. Thus activated sludge flocs were enlarged by this excessive filamentous growth and could easily attach gas bubbles. Due to the hydrophobic cell surface of *M. parvicella* these flocs are extremely sensitive to passive flotation and subsequent formation of stable scum layers. Furthermore it was shown that the hydrophobicity of activated sludge decreased in the presence of fewer numbers and shorter filaments of *M. parvicella* growing to a large extent inside the flocs, producing less stable scum layers. This was observed during the summer season in the present study and after aluminum dosage controlling *M. parvicella*-dominated sludges as is described by Paris and co-workers (2005, Paris et al. 2005). In contrast to *M. parvicella*, nocardioform actinomycetes demonstrated for different *Rhodococcus* spp. and *Dietzia* spp. increase their population density by actively growing in the floated sludge fraction. There they stabilize the scum layer by both hydrophobic cell surfaces and biosurfactant production as was shown by Blackall and Marshall (1989).

In addition to scum bacteria, extracellular polymeric substances (EPS) of activated sludge and scum might play a significant role in scum formation and stabilization. Most reports bring to focus the functional role of EPS in floc aggregation, flocculation, and sludge settleability (for details see chapter 2.2.2). However, little is known so far about EPS composition and its function in scum. Cohesion between EPS molecules and in addition to cell surfaces is described by the DLVO theory for colloidal stability including electrostatical interaction, van der Waals forces, and hydrogen bonds (Zita and Hermansson 1994, Mayer et al. 1999). In addition, hydrophobic interactions between hydrophobic substances and hydrophobic cell surfaces are responsible for a stronger attraction of EPS to cells (Zita and Hermansson 1997b). Cell surface hydrophobicity was reported to be a very important component affecting bacterial cell adhesion to activated sludge flocs promoting floc aggregation (Zita and Hermansson 1997a, Olofsson et al. 1998). Furthermore sorption processes of hydrophobic substances to activated sludge flocs were described by different authors (Finlayson et al. 1998, Hwu et al. 1998, Fang and Zheng 2004) and discussed to be the prerequisite for a successful biodegradation (Hwu et al. 1998, Dueholm et al. 2001). Fang and Zheng (2004) showed that the sorption capacity of organic substances increased with the hydrophobicity of the substances. In general, sorption processes represent the main elimination path for hydrophobic organic pollutants in activated sludge treatment and in biofilm systems (Finlayson et al. 1998, Späth et al. 1998). Therefore EPS might (i) influence scum formation directly by providing hydrophobic sites and (ii) act as a nutrient source providing hydrophobic substances that favor attachment and growth of hydrophobic scum bacteria.

The low TOC concentration detected in the aeration tank water phase in the present study indicates that most of the organic substrates, which enter the system by the primary effluent, is removed by sorption to the activated sludge or scum EPS and/or by degradation. This coincides with the concept of modern nutrient removal WWTPs, representing most of the plants analyzed in the present study, being operated at low sludge loads to support full nitrification. Therefore a low substrate supply is available within the water phase in these plants. This is in contrast to former high loaded activated sludge WWTPs, which are characterized by a high substrate availability within the water phase. Present conditions favor the growth of organisms being adapted to hydrolysis of particulate substrate that are accumulated in activated sludge EPS and scum EPS. Therefore in WWTPs operated under low load sludge condition EPS might play a key role in substrate supply for the microbial

biocenosis. The growth of scum bacteria in the different sludge fractions described might be favored by EPS of activated sludge or scum that can serve as a nutrient source (Wolfaardt et al. 1995). The hydrolysis of exogenous macromolecules by extracellular enzymes, e.g. lipase and protease, provides low-molecular-weight nutrients, representing the key step before substrate uptake and degradation. Therefore extracellular enzyme activity determined in activated sludge and scum indicates the capacity to metabolize EPS by the bacterial biocenosis (Nybroe et al. 1992, Frølund et al. 1995, Schade and Lemmer 2004, 2005).

The EPS components protein and carbohydrate showed different amounts for the various samples, however, the protein to carbohydrate ratio of 2 to 6 was similar for all samples. Neither significant differences between activated sludge and scum nor correlation to hydrophobicity and to the dominant scum bacteria could be detected. In contrast to these findings, other authors (Jorand et al. 1998) demonstrated that EPS proteins contribute to hydrophobicity of activated sludge whereas a high carbohydrate content causes hydrophilic properties. Wilen et al. (2003) showed that both EPS components protein and carbohydrate correlate negatively with sludge hydrophobicity. These observations indicate a strong variation in the physicochemical properties of EPS depending on the origin of the sludge. The measured protease activity (Schade and Lemmer 2004, Müller et al. submitted) demonstrated that a high degradation of protein took place in the sludge EPS. However, no significant correlation between protease activity and the occurrence of scum bacteria was determined. Consequently, a wide range of different organisms within the sludge biocenosis might be able to degrade polymeric and oligomeric proteins. In contrast, lipophilic substances, which can represent up to 30 to 40% of the organic matter in municipal wastewater (Raunkjær et al. 1994), in general showed a positive correlation to the presence of *M. parvicella* and *Rhodococcus* spp. (cluster A and B), which was most clearly observed during the winter and early spring seasons. These lipophilic substances were accumulated during the winter season within the activated sludge EPS in the *M. parvicella*-dominated plant and in the scum EPS in the *Rhodococcus* spp.-dominated plant favoring the growth of these specific scum organisms in the different sludge fractions. This was confirmed by higher lipase and overall respiratory activities within activated sludge as compared to scum in *M. parvicella*-dominated plants (Schade and Lemmer 2004, Müller et al. submitted). Similar results were obtained by in situ analysis (using enzyme labeled fluorescence-technology, ELF) demonstrating that *M. parvicella* exhibited lower lipase activity in scum as compared to activated sludge, even at high population densities (Schade and Lemmer 2004, 2005). In contrast, a higher lipase activity in scum as compared to activated sludge (Schade and Lemmer 2004, Müller et al. submitted), corresponding to a higher respiratory activity in scum, was determined for *Rhodococcus* spp.-dominated scum indicating that these species might utilize fatty acid esters as carbon sources. This was confirmed by in situ lipase activity measurments detecting high numbers of lipase-active *Rhodococcus* sp. cluster B in the scum layer (Schade et al. 2006). For these scum samples, the direct effect of EPS on the hydrophobic properties of the scum layer was probably caused by lipophilic substances. This confirmed the second role of EPS apart from nutrient supply: EPS provide hydrophobic sites within the scum matrix that increase scum hydrophobicity and support the development of a stable scum layer. No detection of lipophilic substances during late spring and summer indicates that lipophilic substances might be more rapidly degraded during these seasons as compared to the winter period, possibly due to a higher solubility of lipids and fatty acids and a higher bacterial

metabolic activity during these periods. This is confirmed by a higher lipase activity measured for *M. parvicella* during spring as compared to the winter period (Schade and Lemmer 2004, Müller et al. submitted).

The growth of *Rhodococcus erythropolis* could not be attributed to the presence of lipophilic substances and lipase activity (Schade and Lemmer 2004, Müller et al. submitted), and consequently these organisms might metabolize other organic substances than triglycerides and fatty acids. The high residual fraction of TOC (approximately 50%) in the EPS beside proteins, carbohydrates, and lipophilic substances might provide, e.g. refractory organic substances (humic substances) or highly adsorbable xenobiotics, which favor the growth of this organism. These observations are consistent with the knowledge that nocardioform actinomycetes are able to utilize a wide range of different carbonaceous substrates beside readily degradable as well poorly degradable hydrocarbons. The latter enhance the competitive growth of nocardioform actinomycetes in the presence of faster-growing organisms (Lemmer and Baumann 1988a). For *Rhodococcus erythropolis* high growth rates on n-alkanes are reported in the literature (Soddell et al. 1998). In contrast to the different *Rhodococcus* spp., *Dietzia* spp. found in the present study were not able to metabolize high molecular weight substrate as no exoenzyme activity could be assigned to these nocardioform actinomycetes species demonstrated by Schade and Lemmer (2006). Hence *Dietzia* spp. might be more dependent on soluble carbon fraction, which explain their high growth rate in scum layer floating on the surface of the high loaded aeration tank of a two-stage WWTP. Genera- and species-dependent preferences for different substrates found in the present study and in the literature require reliable taxonomic determination before specific troubleshooting measures against nocardioform actinomycetes can be performed. In contrast to nocardioform actinomycetes, *M. parvicella* is known to have a very limited substrate spectrum with C16/C18 long-chain fatty acids and their esters and reduced nitrogen sources only (Slijkhuis 1983a, Andreasen and Nielsen 1998, 2000). This corresponds well with the higher lipase activity analyzed for *M. parvicella*-dominated sludges in comparison to nocardioform actinomycetes-dominated sludges, demonstrating that *M. parvicella* is more active on long chain fatty acids (Schade and Lemmer 2004, Müller et al. submitted).

The results of the present study show clearly that both hydrophobic scum bacteria in particular *M. parvicella* and nocardioform actinomycetes, and activated sludge EPS and scum EPS effectively contribute to the development of a stable scum layer. Specific hydrophobic EPS components such as triglyceride esters are promoting the growth of these specific bacteria producing hydrophobic cell surfaces to improve the attachment to hydrophobic carbon sources before substrate uptake and metabolizing. These hydrophobic cell surfaces and also hydrophobic sites within the EPS are increasing sludge hydrophobicity, causing stable scum layers. However, *M. parvicella* and the nocardioform actinomycetes, *Rhodococcus* spp. and *Dietzia* spp., were shown to grow in different sludge fractions and lead to scum development in two different ways. *M. parvicella* increases hydrophobicity of the activated sludge fraction followed by passive flotation of the biomass to the water surface. In contrast, nocardioform actinomycetes stabilize scum layers by growing directly in this fraction. Therefore the investigation of the activated sludge biocenoses in WWTPs without scum events reveals information about the scum potential in *M. parvicella*-dominated plants because scum development is directly influenced by the activated sludge biocenosis. In contrast, in nocardioform actinomycetes-dominated plants the excessive growth of these

organisms is only detectable in scum and stabilizes this fraction. Therefore the activated sludge bacterial community does provide limited information about the scum process only.

The detected metabolic differences found for nocardioform actinomycetes- and *M. parvicella*-dominated sludges lead to different control strategies. Nocardioform actinomycetes, most active and able to grow in the scum layer, are to be controlled in this fraction. In contrast, *M. parvicella*, growing in the activated sludge fraction, needs to be controlled right there. On the basis of these observations different troubleshooting strategies have to be performed depending on which organisms dominate the bacterial biocenosis. The treatment of activated sludge with aluminum salts is successfully applied when high numbers of *M. parvicella* are detected. This treatment reduces the amount of filamentous *M. parvicella* by inhibiting their lipase activity (Eikelboom et al. 1998, Lind and Lemmer 1998, Paris et al. 2005, Schade and Lemmer 2005). In most WWTPs with predominance of nocardioform actinomycetes the scum layer is to be continuously removed manually or by a scum harvester. In this case it is crucial the floated scum to be not recycled in the system but to be deposited elsewhere (Eikelboom 2000). Since optimal growth rates of various nocardioform actinomycete species are determined by different environmental conditions, more specific troubleshooting measures, based upon a reliable taxonomic determination at genera and species level, are required for nocardioform actinomycete-dominated scums. Furthermore troubleshooting measures for plants dominated by both *M. parvicella* and nocardioform actinomycetes might include limiting lipids and long- chain fatty acid supply by controlling the primary effluent, avoiding high sludge age with a concomitant sludge/cell lysis and biotenside production (Lemmer et al. 2002).

7 CONCLUSIONS

The main findings of this study including identification of scum bacteria populations and their key role along with extracellular polymeric substances (EPS) in scum development can be concluded as follows:

1. Screening investigations of the scum biocenosis using classical microscopic sludge analysis revealed that *Microthrix parvicella* represents the most frequently detected bacterium followed by nocardioform actinomycetes. Type 1863, which has not been previously described as a typical scum bacterium, occurred in several WWTPs enriched in the scum fraction but was never found to dominate the bacterial scum biocenosis. Other filamentous morphotypes such as type 0041/0675, 1851, 0092, and *N. limicola* were detected in distinctly lower numbers and appeared never enriched in the scum fraction. Consequently, these microorganisms might be less involved in scum formation.

2. The results of classical microscopy and FISH correspond well in case of filamentous *M. parvicella*, types 1863, and 1851. These findings indicate that these morphotypes are represented by clear phylogenetic entities identified as "*Candidatus* Microthrix parvicella", an unclassified member of the *Actinobacteria*, *Acinetobacter* spp. and a member of the *Chloroflexi* subdivision 3, respectively. Typical branched filamentous nocardioform actinomycetes were identified by both classical microscopic sludge analysis and FISH. However, FISH underestimates the number of nocardioform actinomycetes in some samples due to insufficient cell wall permeability. In contrast, classical microscopy failed to detect non-branched filamentous and non-filamentous nocardioform actinomycetes, and coccoid *Acinetobacter* spp.. Furthermore identification of nocardioform actinomycetes at genus and species level was limited by both methods. Most nocardioform actinomycetes develop the typical branched morphotype and consequently classical microscopic sludge analysis is not able to differentiate between these species. FISH did not succeed in identifying most nocardioform actinomycetes at species level because of the lack of adequate probes. A high taxonomic variability was determined for morphotypes 0041/0675, 0092, and *N. limicola* by FISH only. However, final identification of these morphotypes was limited by FISH because of the deficit of specific probes. This in general causes a lower detection frequency by FISH as compared to classical microscopic sludge analysis. Therefore, to obtain reliable detection results, the application of both classical microscopic sludge analysis and FISH is recommended to compensate for the limitation of either technique. However, only a sound taxonomic identification will reveal information about the physiological properties of these scum bacteria, which might be helpful for the understanding of the scum development process and the application of specific control measures.

3. A high diversity within the nocardioform actinomycete group (*Corynebacterineae*) was revealed after the application of different molecular biological methods. It could be shown that different species, e.g. *Dietzia* spp., *Gordonia* spp., *Rhodococcus* spp., and *Skermania piniformis*, are associated with scum formation. The full-cycle 16S rRNA approach proved extremely useful to identify and quantify unknown nocardioform actinomycete species dominating various scum samples. The combination of different molecular biological tools

to investigate clone libraries and environmental DNA eased the selection of clone sequences within the different clones libraries that might dominate the original bacterial biocenosis. The screening with *Actinobacteria*-specific primers reduced the microbial diversity within the clone libraries and environmental DNA. This was the prerequisite to group the clones into taxonomic units and to screen the clone libraries for redundancy by nucleic acid fingerprinting methods such as RFLP and DGGE. However, only the analysis of full length sequences of clone inserts followed by probe design and the application of FISH using specific probes for *Dietzia* spp. and different *Rhodococcus* spp. were able to verify and quantify these organisms within the microbial scum communities.

4. FISH quantification of samples from German WWTPs with scum events showed that different *Rhodococcus* spp. (*R. erythropolis* and probably two new *Rhodococcus* species, characterized by the typical branched filamentous morphotype) were detected in high numbers in the scum fraction and were often accompanied by *Dietzia* spp. growing as non-branched short filaments or in cell clusters (generally found in lower numbers). In contrast, scum samples from Australian WWTPs were dominated by *Gordonia* spp. and *Skermania piniformis*. The different growth conditions concerning temperature and the availability of different carbon sources typical for the WWTPs analyzed might be responsible for these variations.

5. The correlation of quantitative FISH data of the scum bacteria *M. parvicella* and nocardioform actinomycete species with physicochemical parameters such as hydrophobicity and EPS composition provided knowledge about scum development. The characterization of EPS showed that lipophilic substances originating from the primary effluent and being adsorbed from the water phase to the EPS play two major roles in the scum process: (i) they increase hydrophobicity directly and (ii) they support the growth of hydrophobic organisms. In contrast, protein and carbohydrate content within the EPS showed no correlation with sludge hydrophobicity and the occurrence of scum bacteria. The high residual TOC content in EPS pointing to a substrate pool beside proteins, carbohydrates, and lipophilic substances. This substrate pool might include refractory organic substances (humic substances) or else highly adsorbable xenobiotics, which might support the growth of specific scum bacteria populations.

6. In the case of *M. parvicella* lipophilic substances within the activated sludge EPS cause good nutrient conditions and promote the growth of *M. parvicella* right in this fraction. Thus the filamentous growth of *M. parvicella* enlarged the activated sludge flocs and made these open flocs passively float to the water surface due to gas trapped at the hydrophobic cell surfaces of this organism. This observation was confirmed by both lower lipase and respiratory activity in scum as compared to activated sludge, even at high population densities.

7. In contrast to *M. parvicella*, nocardioform actinomycetes identified as several *Rhodococcus* species and *Dietzia* spp. with their higher respiratory activity in scum as compared to activated sludge might increase their population density by actively growing in the floated sludge fraction.

8. The ability of nocardioform actinomycetes to metabolize different substrates is genera- and species-dependent. High numbers of *Rhodococcus* spp. A and B, which probably represent new species within the genus *Rhodococcus*, were positively correlated with lipophilic substances within the scum EPS and lipase activity. Thus these species are likely to be active on lipids and long chain fatty acids degradation in the scum layer. Both lipophilic substances and cell surface properties of these species caused hydrophobicity right in this fraction and thus stabilized the scum layer. In *Dietzia*- and *Rhodococcus erythropolis*-dominated scum no or a very low amount of lipophilic substances were detected in the EPS. Thus these organisms might be able to metabolize other substrates beside carbohydrate, proteins and lipophilic substances. This might be provide by the high residual non-characterized TOC fraction of the scum EPS in the case of *Rodococcus erythropolis*-dominated scum. In contrast, *Dietzia* spp. favor to grow on soluble carbon fraction explaining their high growth rate inWWTPs with high sludge load.

9. The described growth strategies and scum processes, which are dependent on the scum bacteria involved in scum formation, demand different control measures. *M. parvicella* growing in the activated sludge fraction has to be controlled directly in this fraction. In contrast, nocardioform actinomycetes, which are most active and able to grow in the scum layer, are to be controlled in this particular fraction.

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9 APPENDIX

9.1 rRNA-targeted Oligonucleotide Probes

Table 20: rRNA-targeted oligonucleotide probes used for FISH with target sites and probe sequences.

oligonucleotide probe	target site (rRNA position)[a]	sequence (5´-3´)[b]	reference
EUB338 EUB338-II EUB338-III = EUB338-mix	16S rRNA 338-355	GC**T**GCC**T**CCCGTAGG**A**GT GC**A**GCC**A**CCCGTAGG**T**GT GCTGCC**A**CCCGTAGGTGT	(Amann et al. 1990) (Daims et al. 1999)
ACA23a	16S rRNA 652-669	ATCCTCTCCCATACTCTA	(Wagner et al. 1994)
HGC69a	23S rRNA 1901-1918	TATAGTTACCACCGCCGT	(Roller et al. 1994)
HGC1156	16S rRNA 1156-1173	CGAGTTGACCCCGGCAGT	(Erhart 1997)
HGC235 HGC270H	16S rRNA 235-253 16S rRNA 270-287	AACAAGCTGATAGGCCGC ACCCGTCG**HM**GCCTTGGT	(Erhart 1997) (Glöckner et al. 2000)
MPA60 MPA223 MPA645 = MPA-mix	16S rRNA 60-77 16S rRNA 223-240 16S rRNA 645-661	GGATGGCCGCGTTCGACT GCCGCGAGACCCTCCTAG CCGGACTCTAGTCAGAGC	(Erhart et al. 1997)
MNP1	16S rRNA 152-172	TTAGACCCAGTTTCCCAGGCT	(Schuppler et al. 1998)
Myc657	16S rRNA 657-673	AGTCTCCCCTG**Y**AGTA	(Davenport et al. 2000)
CMN119	16S rRNA 119-136	GGCAGATCACCCACGTGT	(Erhart 1997)
G-Gor-0596-a-A22	16S rRNA 596-617	TGCAGAATTTCACAGACGACGC	(de los Reyes et al. 1997)
NPI425	16S rRNA 452-492	GAAGGTACCGACACCCCGAAAGGCT	(Bradford 1997)
NLIMI 91	16S rRNA 91-108	CGCCACTATCTTCTCAGT	(Liu and Seviour 2001)
NLIMII 175	16S rRNA 175-192	GGCTCCGTCTCGTATCCG	(Liu and Seviour 2001) (Bradford 1997)
NLII65	16S rRNA 65-83	CAAGCTCCTCGTCACCGTT	
AHW183	16S rRNA 183-200	CCGACACTACCCACTCGT	(Schade et al. 2002)

Table 20 continued.

oligonucleotide probe	target site (rRNA position)[a]	sequence (5´-3´)[b]	reference
PPx3-1428	16S rRNA 1428-1447	TGGCCCACCGGCTTCGGG	(Snaidr et al. 2002) (Levantesi et al. 2004)
Noli-644	16S rRNA 644-660	TCCGGTCTCCAGCCACA	
MC2-649	16S rRNA 649-667	CTCTCCCGGACTCGAGCC	
NLIMIII 301	16S rRNA 301-318	CCCAGTGTGCCGGGCCAC	(Liu and Seviour 2001)
0092-997	16S rRNA 997-1018	ATTTCTAAATCTGTCGAATCCC	(Bradford 1997)
CHL 1851	16S rRNA 592-612	AATTCCACGAACCTCTGCCA	(Beer et al. 2002)
TM7305	16S rRNA 305-322	GTCCCAGTCTGGCTGATC	(Hugenholtz et al. 2001)
TM7905	16S rRNA 905-926	CCGTCAATTCCTTTATGTTTTA	

[a] from E.coli numbering of Brosius et al. (1981)

[b] using oligonucleotide IUB-codes

9.2 Media used for Pure Culture and Cloning Approach

The sterilization of the described media was carried out by autoclaving at 121°C for 20 min. Agar plates were prepared by adding 15g L^{-1} agar to the liquid media.

R2A medium (1 l)

Yeast extract	0.50 g
Proteose peptone	0.50 g
Casamino acids	0.50 g
Glucose	0.50 g
Soluble starch	0.50 g
K_2HPO_4	0.30 g
$MgSO_4$ x7H_2O	0.05 g
Sodium pyruvate	0.30 g

H_2O_{bidest} add up to a final volume of 1000 ml
pH 7.0

SOC medium (100 ml)

Bacto-tryptone	2.0 g
Bacto-yeast extract	0.5 g
1 M NaCl	1.00 ml
1M KCl	0.25 ml
[a] 2M Mg^{2+}	1.00 ml
[a] 2M glucose	1.00 ml

H_2O_{bidest} add up to a final volume of 100 ml
pH 7.0

[a] 2 M Mg^{2+} and 2 M glucose, both stock solutions were filter-sterilized and added to the medium (final concentration of 20 mM) after autoclaving.

LB medium with ampicillin (1 l)

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	5 g
[a] Ampicillin (100 mg ml^{-1})	1 ml

H_2O_{bidest} add up to a final volume of 1000 ml
pH 7.0

[a] After autoclaving the medium was cooled to 50°C before adding ampicillin to a final concentration of 100 µg ml^{-1}.

DSMZ medium 53, Corynebacterium medium (1 l)

Casein peptone, tryptic digest	10.0 g
Yeast extract	5.0 g
Glucose	5.0 g
NaCl	5.0 g

H_2O_{bidest} add up to a final volume of 1000 ml
pH 7.2 - 7.4

DSMZ medium 65, GYM Streptomyces medium (1 l)

Glucose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
$CaCO_3$	4.0 g

H_2O_{bidest} add up to a final volume of 1000 ml
pH 7.2 (adjust with KOH)

DSMZ medium 354, Löwenstein-Jensen medium (1 l)

KH_2PO_4	2.50 g
$MgSO_4$	0.24 g
Mg-citrate	0.60 g
L-asparagin	3.60 g
Potato flour	30.00 g
Malachite green	0.40 g
Glycerol	12.00 ml
[a] Fresh egg mixture	1000.00 ml

H_2O_{bidest} add up to a final volume of 1000 ml
pH 7.0

[a] After autoclaving the medium was cooled to 30°C before adding fresh egg mixture

9.3 List of Abbreviations

ARDRA	Amplified Ribosomal DNA Restriction Analysis
BLAST	Basic Local Alignment Search Tool
BOD_5	5-day Biological Oxygen Demand [mg L^{-1}]
CLSM	Confocal Laser Scanning Microscope
CM	Classical Microscopy = microscopic sludge analysis of filamentous bacteria
CTC	5-Cyano-2,3-ditolyl Tetrazolium Chloride
CTF	5-Cyano-2,3-ditolyl Tetrazolium Formazan
DGGE	Denaturing Gradient Gel Electrophoresis
EBPR	Enhanced Biological Phosphorus Removal
EPS	Extracellular Polymeric Substances
FISH	Fluorescence In Situ Hybridization
F/M ratio	Food to Microorganism ratio = sludge load [kg BOD_5 kg^{-1} MLSS d^{-1}]
GALO	*Gordonia amarae*-Like Organisms
LCFA	Long-Chain Fatty acid
MATH	Microbial Adhesion To Hydrocarbons
MAC	Microspheres Adhesion to Cells
MLSS	Mixed Liquor Suspended Solids

NALO	*Nocardia amarae*-Like Organisms / NocArdioform-Like Organisms
NOC	NOCardioform actinomycetes
PAO	Phosphorus Accumulating Organisms
PCR	Polymerase Chain Reaction
PE	Population Equivalents
PTLO	Pine Tree-like Organisms
RAS	Returned Activated Sludge
rDNA	ribosomal DeoxyriboNucleic Acid
RDP	Ribosomal Database Project
RFLP	Restriction Fragment Length Polymorphism
rRNA	ribosomal RiboNucleic Acid
SS	Suspended Solids
TOC	Total Organic Carbon
VS	Volatile Solids
WWTP	Wastewater Treatment Plant

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