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Molecular and microscopic studies on the diversity of protozoa, bacteria and fungi and their impact on the structural formation of aerobic sewage granules

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Abbreviations

AOB	ammonia oxidizing bacteria
ARB	software environment for sequence data (latin, "arbor"=tree)
bp	basepair
°C	degree Celsius
CLSM	confocal laser scanning microscopy
DNA	desoxyribonucleic acid
EMBL	European Molecular Biology Laboratory
EPS	extracellular polymeric substances
FISH	fluorescence in situ hybridization
h	hour
ITS	internal transcribed spacer
kb	kilobases
M	molar
µg	microgram
µm	micrometer
µM	micromolar
mg	milligram
ml	milliliter
min	minute
ng	nanogram
nm	nanometer
NOB	nitrite oxidizing bacteria
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
SBR	sequencing batch reactor
SEM	scanning electron microscopy
WWTP	wastewater treatment plant

Original Publications

Most of the fundamental results derived from this study together with the corresponding discussion, conclusions, materials and methods are described in detail in the publications listed below. The original articles and the respective author contributions can be found in the section Appendix A-C.

The symbol ➲ at the beginning of a chapter designates that the chapter content was published as part of the indicated appendix/publication.

Appendix A **Weber, S. D., Wanner, G., Ludwig, W., Schleifer K. H., and Fried J.** (2007). Microbial composition and structure of aerobic granular sewage biofilms. *Appl. Environ. Microbiol.* 73: 6233-6240.

Appendix B **Weber, S. D., Hofmann, A., Pilofer, M., Wanner, G., Agerer, R., Ludwig, W., Schleifer K. H., and Fried J.** (2009). The diversity of fungi in aerobic sewage granules assessed by 18S rRNA gene and ITS sequence analyses. *FEMS Microbiolgy Ecology 68(2): 246-254.*

Appendix C **Weber, S. D., Schwarzenbeck, N., Lemmer, H., Wanner, G., Ludwig, W., Schleifer K. H., and Fried J.** (submitted). Diversity, population dynamics and association of protozoa and bacteria in aerobic sludge granules from industrial wastewater sequencing batch reactors. *Submitted to FEMS Microbiology Ecology.*

A General Introduction

A.1 Sequencing batch reactors (SBR) in wastewater treatment

Wastewater purification using activated sludge and simulating the natural purification process of water as observed in rivers or other watercourses was first established 1914, by Ardern and Lockett in Salford, UK. Since then the use of activated sludge has been the most common wastewater treatment technique (Miksch and Fingerhut, 1990; Tchobanoglous and Burton, 1991). For the past several decades, treatment techniques were modified and many different application forms besides common aeration/sedimentation tanks were used such as trickling filters, membrane bioreactors, rotating biological contractors, and, finally, the sequencing batch reactor (SBR). The SBR technique had already been proposed at the end of the 19th century by the English engineer Sir Thomas Wardle, but was not rediscovered until 1952 by Hoover and Porges and in 1959 by Pasveer. It was finally introduced by Irvine et al. in 1977. Since then it has continuously been enhanced and is at present increasingly operated with a special form of activated sludge condensed to granular biofilms. For a number of reasons this combination has turned out to be one of the most exciting and effective treatment tools in wastewater purification processes.

Sequencing batch reactors are cylindric aeration tanks in which the purification process is managed along a time axis, as opposed to conventional aeration tanks using a road axis. This implies that all phases during purification take place in the same basin, as shown in Fig. 1, and are not separated spatially. The length of treatment phases can be adapted according to specific requirements.

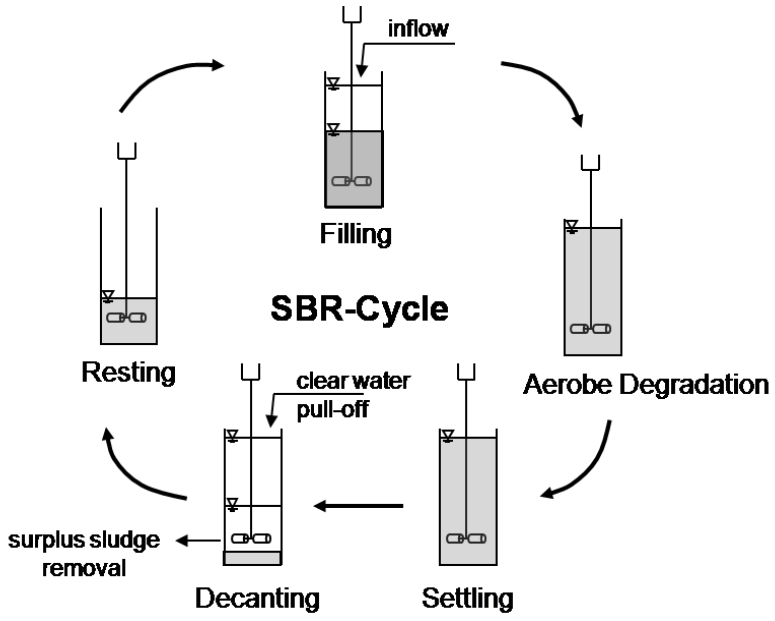


Fig. 1: Wastewater treatment phases in the SBR cycle

Sequencing batch reactors provide many advantages compared to common flow-through aeration tanks. The most important are:

• Reduced construction effort, small footprint and minimal operating costs
• Very high purification efficiency
• Sedimentation and withdrawal take place on ideal, i.e. influent-free conditions
• The fully automated operation strongly facilitates management of the activated sludge plant
• SBRs are odorless and can be operated in settlement areas
• Minimal place requirement compared to common aeration tanks due to their vertical buildup as columns

Figure 2 shows two lab-scale SBRs connected to fully automated timer systems which control the entire purification process. The left of the displayed SBRs is filled with aerobic granules and malthouse wastewater and was used in this study amongst other SBRs for the examination of granular biofilms.

By adjusting the biomass retention time, i.e. the remaining sludge volume in the reactor relating to the removed sludge volume per day, the medium growth rate limit for organisms to stay in the reactor can be set. A high sludge age provides an opportunity for slow growing organisms to hold their ground in the reactor, and the formation of and selection for granules can specifically be actuated.

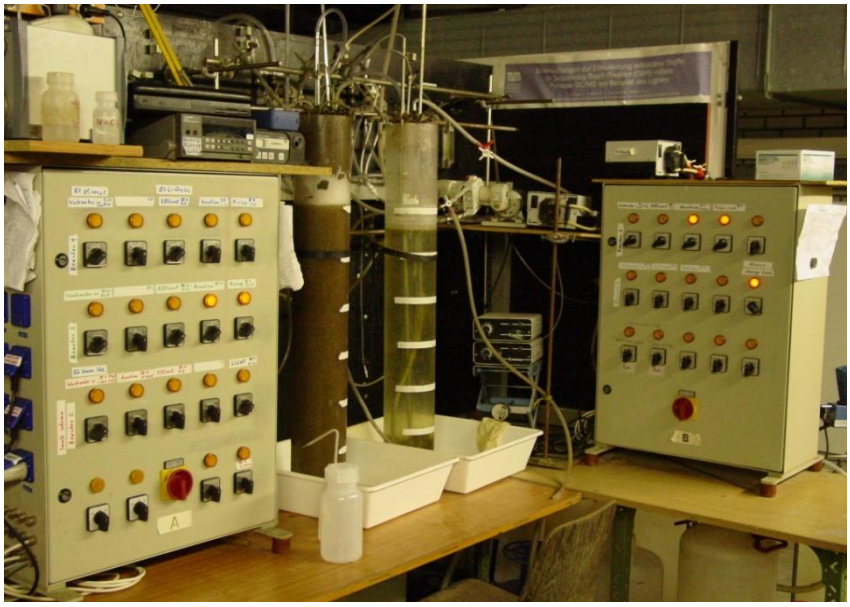


Fig. 2: Lab-scale SBR system with fully automated timers, used in this study. Left SBR filled with aerobic sludge granules and malthouse wastewater during aeration phase.

In this study, five SBRs with a set-up as displayed in Fig. 2 were used for the investigation of granular sludge. They were operated with dairy, malthouse, brewery and synthetic wastewater.

A.2 Aerobic sludge granules

Granular sludge was first described in upflow anaerobic sludge blanket reactors (UASBR) (Lettinga et al., 1980), and since then large-scale UASBRs were operated mainly with highly concentrated wastewater. Later on, spontaneous granule formation was also discovered in aerobic SBRs, providing the opportunity to perform important aerobic processes in SBRs, e.g. nitrification. Aerobic granules were first described by van Loosdrecht et al. (1997). Several studies followed in the next years (Morgenroth et al., 1997; Etterer and Wilderer, 2001) and supported the implementation of this technique in common activated sludge treatment.

Aerobic sewage granules are spherical, compact aggregates of microorganisms, mainly bacteria, but also protozoa and fungi, and extracellular polymeric substances (EPS) (Morgenroth et al., 1997; Beun et al., 1999; Wang et al. 2004; Weber et al., 2007; Lemaire et al., 2008; Weber et al., 2009). They are considered to be a special biofilm formation, composed of self-immobilized cells building a "biofilm in suspension".

Granular sludge formation is enhanced in SBRs by high hydraulic shear rates and frequent changes of substrate availability. It is also enhanced by very short sedimentation and draw phases, which lead to the washout of slow settling biomass and promote granules selection. At least a completely grained biomass is obtained in the reactor.

The granulated biomass comprises many advantages compared to conventional activated sludge flocs (Morgenroth et al., 1997; Venugopalan et al. 2005; Zheng et al. 2006; Wang et al. 2007). Due to their compact and dense structure, granular biofilms allow faster settling of the sludge, whereby the biomass is almost completely retained in the system and features a high and stable metabolism and conversion rates. CSB elimination is achieved up to 99% with a simultaneous N-elimination of up to 80% with respiration rates being 4-5-fold higher than in flocculent sludge (Schwarzenbeck, pers. comm.). The original efficiency after anaerobic storage for several weeks can be replicated within 1-2 days (Schwarzenbeck, pers. comm.). The compact form enables microorganisms inside the granules to withstand fluctuating organic loading rates which arise quite often in real-life remediation scenarios. Additionally, granular biofilms exhibit a very good resilience to toxin shocks due to protection by their EPS matrix (Wingender et al., 1999).

Due to the permanent immobilization of biomass inside the aggregates, granular sludge provides long biomass residence times. Thus, granules might function as carriers of microorganisms with specific catabolic information and could therefore be used for bioaugmentation of wastewaters containing xenobiotics (Bathe et al., 2004).

Various investigations on general characteristics of sludge granules, such as size, structure, settling performance, stability against shear forces, EPS content, reactor performance and metabolism rates, were previously performed. Theories about the crucial factors of granule development have been intensively discussed (Etterer and Wilderer, 2001; Moy et al., 2002; Wilderer et al., 2004; Hulshoff et al., 2005; de Kreuk, et al., 2005; Inizan et al., 2005, McSwain et al., 2005). However, the possible role of eukaryotes (i.e. protozoa and fungi) in the biofilm formation process and their accompanying interaction with bacteria has so far been neglected. Simply the presence of these organisms in granules was mentioned in a few studies (Beun et al., 1999; Etterer and Wilderer, 2001; Wang et al., 2004).

The present study investigated the structural development of aerobic granules, focusing on the role and diversity of protozoa and fungi. Furthermore, the associations and interactions between these eukaryotes and bacteria were examined. Thus, this study enriches the research in modern wastewater treatment by adding new aspects and providing a deeper insight into interspecies microbial communities for a more all-encompassing understanding of aerobic granular biofilms.

A.3 Protozoa in wastewater treatment

Protozoa are ubiquitous organisms which can live in clear headstreams as well as in feculent wastewater. Temperature, oxygen, light incidence, salt content, pH-value, and fluid flow are some significant factors which determine the protozoan species composition of a particular habitat.

In activated sludge systems protozoa generally constitute a natural part of the microbial community and are known to fulfill a wide variety of important tasks in biomass conversion, e.g. mineralization, and water clarification processes (Curds et al., 1968; Curds, 1982; Madoni, 1991; Ratsak et al., 1994; Martin-Cereceda et al. 1996; Perez-Uz et al., 1998; Nicolau et al., 2001, Fried and Lemmer, 2003).

For quite some time they have been used as bioindicators for water quality, the health of sludge and treatment efficiency (Curds and Cockburn, 1970; Guhl, 1985; Madoni, 1994; Martin-Cereceda et al., 1996; Fried et al., 2000). They can be regarded as indicators of pollution due to their presence or absence related to particular environment conditions. The genus *Epistylis* was especially identified as an important bioindicator organism (Sladacek,

1986). Furthermore, populations of several ciliate species are considered as test organisms to evaluate the toxicity of relevant toxic compounds (Nicolau et al., 2001).

In contrast to bacteria, protozoa prefer the uptake of suspended solids. This enhances the clarification of water significantly as shown in quite a number of studies (e.g. Curds and Cockburn, 1970; Schwarzenbeck et al., 2004; Lemaire et al., 2008). Protozoa also ingest single bacterial cells (Guhl, 1985; Iriberri et al., 1994; Westermeier et al., 2006) and control bacterial growth by grazing (Gonzalez et al., 1990). Amongst others, this was regarded as a very useful and cost effective method for the removal of antibiotic resistant bacteria from sludge (Eichler et al., 2006). Roessink and Eikelboom (1997) showed that large ciliate populations consume more than 99% of free bacterial cells and increased the removal efficiency from less than 10% up to 15 - 25%. In another study of Eisenmann et al. (2001), approximately 14% of the water had been clarified solely by ciliate filter feeding.

An interesting aspect is that protozoa can excrete not only growth factors for bacteria (Ratsak et al., 1996) but also extracellular polysaccharides which support floc and biofilm aggregation (Arregui et al., 2007). Of additional importance, with respect to the support of biofilm formation by ciliates, is their influence on the nutrient flux towards and into the biofilm due to their cilia movement (Hartmann et al., 2007). All of these facts indicate that protozoa and ciliates especially play an important role for the interactions within the activated sludge community.

Besides the already known significant roles of these organisms, new aspects arise with the development of aerobic granular wastewater treatment techniques. Some protozoa might influence biofilm formation and structure more deeply than previously assumed. On this note, the present study investigates the diversity and role of stalked ciliates in granule formation. Within that scope a further focus is set on their population dynamics during the granule formation process.

A.4 Bacteria in wastewater biofilms

Bacteria are mainly responsible for biomass conversion in activated sludge treatment systems, i.e. the degradation of carbonaceous substrates under aerobic and anaerobic conditions, nitrification, denitrification and phosphorus removal processes (Fig. 3; Lemmer, 2000, Schmid, 2002). The nitrification process is an aerobic two-step procedure which includes the oxidation of ammonia to nitrite, followed by the oxidation of nitrite to nitrate. Denitrification completes nitrogen elimination by reducing nitrate to molecular nitrogen. The conversion of ammonia and nitrate to nitrogen is essential since release of treated wastewater, containing high anorganic nitrogen compounds concentrations, into nature causes eutrophication and could have toxic effects on ecosystems.

Ammonia and nitrite oxidizing bacteria (AOB, NOB) usually belong to specific taxonomic groups such as *Nitrosomonas* sp.(AOB) or *Nitrospira* sp. (NOB).

The denitrification process is strictly anaerobic and comprises the stepwise reduction of nitrate to molecular nitrogen ("nitrate respiration"). Denitrification can be realized by various microorganisms belonging to more than 100 phylogenetic taxa (Zumft, 1992).

The removal of phosphorus was achieved by chemical precipitation for a long time. For the last decade, the biological process of enhanced biological phosphorus removal (EBPR) has been increasingly implemented (Lemmer, 2000). It is, however, concurrently in the focus of intensive research since the detailed process is not yet fully understood (e.g. Ahn et al. 2007; Oehmen et al. 2007).

In addition to these common purification processes, bacteria are capable of many other degradation pathways for specific substances being present particularly in industrial wastewaters. The microbial activated sludge community will specifically adapt to a specific type of wastewater. Thus, the growth and establishment of bacteria with specific metabolic capabilities in the sludge community is promoted.

During the last twenty years, the closer identification of the mostly non-cultivable microorganisms in activated sludge has become possible by the introduction of molecular methods, i.e. fluorescence in situ hybridization (FISH). The community composition of common wastewater treatment plants (WWTPs) with a low food/microorganism ratio (F/M ratio) normally exhibits a large amount of *Betaproteo-* and *Alphaproteobacteria* (Lemmer, 2000). The use of in situ micro-autoradiography techniques (MAR; e.g. Andreasen and Nielsen 1997, Lee et al., 1999) in combination with FISH (FISH-MAR) also enables the

assignment of specific metabolic functions to different groups of microorganisms. The combined knowledge of taxonomic identity and metabolic function allows an improved characterization of the bacterial sludge community. A more detailed knowledge of the sludge community composition of a distinct WWTP is the basis for monitoring sludge health and avoiding breakdown events. In general, it is known that treatment plants with a high bacterial diversity within a functional group exhibit higher process stability. Furthermore, while investigating bacterial community compositions, specific associations within this community can be examined and useful composition models for specific treatment purposes might be developed. However, for the control of such processes intensive research is still necessary.

In the SBR granulation process bacteria aggregate to biofilms: to avoid nutrient limitations, to gain a better protection to shock loads, pH value drops, toxic compounds and other harmful environmental influences, to resist shear forces and to escape washout. During biofilm formation they are embedded in a matrix of extracellular polymeric substances (EPS) which functions concurrently as sticking glue for the biofilm community. Within this matrix, the share of metabolic information between the microbial members takes place due to small communication molecules released during quorum sensing processes. However, especially aerobic granular biofilms include not only bacteria but also protozoa and fungi. The clustering of the prokaryotes and eukaryotes was found to be very tight, although their distinct associations have so far been scarcely investigated..

In the scope of this study, basic essentials of the bacterial composition within aerobic granules derived from different SBRs during different development phases were determined as a first step. Future studies will elucidate more details on the association of the detected bacterial taxa with ciliates and fungi from similar habitats.

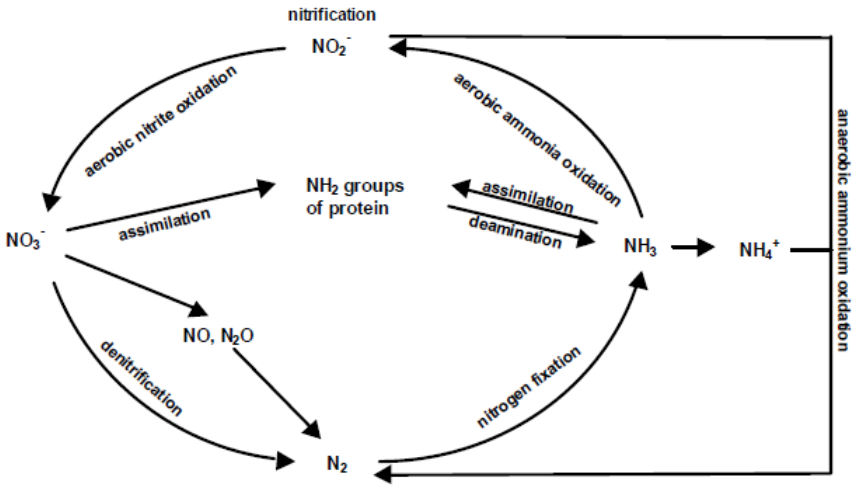


Fig. 3: Extended nitrogen cycle (Schmid, 2002)

A.5 Fungi in wastewater systems

Fungi comprise a major part of living organisms on earth. The total species number is estimated to exceed 1.5 million (Hawksworth, 2001). Of about 70,000 species, functional ecology of only 5% is known. Yet fungi attract more and more attention due to their manifold roles in environmental systems (Bornemann and Hartin, 2000; Hawksworth, 2001). Most investigations on the importance of fungi in wastewater processes highlighted single species which featured the enzymatic treatment of problematic wastewater components. These included white-rot fungi degrading lignin or fungi acting as metal biosorbents due to the properties of their cell wall (Siegel et al., 1990; Dhawale et al., 1996; Yan and Viraraghavan, 2003; Wu et al., 2005). Toxic matters such as chlorinated xenobiotic molecules, mercury, dye substances, phenolic compounds, or polyaromatic hydrocarbons are examples of some common compounds of wastewater released from textile and color industries, olive mills, the coffee industry and alcohol distilleries. Often these substances cannot be metabolized by the bacterial activated sludge community but can be accumulated, degraded, or decolorized by several fungi, e. g. *Trametes versicolor* (Dhawale et al., 1996; Reddy et al. 1998; Yesilada et al., 1999; Robles et al., 2000; Borchert and Libra, 2001; Shin, 2004; Chairattanamanokorn et al., 2005).

However, aside from the use of particular fungi for industrial wastewater treatment, the overall fungal diversity in activated sludge remained mainly unidentified since these organisms were generally unwanted in conventional activated sludge processes. The filamentous hyphae were known to cause bulking problems and clog tubes and filters in the plants (Subramanian et al., 1983; Mudrack and Kunst, 1988). Recent studies, however, also reported on the useful implementation of fungi in domestic wastewater treatment (Guest et al., 2002; Molla et al., 2002). Fungi contributed to an improved sludge dewaterability and toxic resistance, COD removal, nitrification and denitrification (Guest et al., 2002; Alam et al., 2003; Mannan et al., 2005). Some of them show even higher denitrification rates than bacteria (Guest et al., 2002). These observations endorse the need to investigate the fungal diversity in wastewater more closely, and to integrate these organisms systematically into the treatment process in order to tap their possible bioconversion potential without bulking problems. For the latter task, the use of novel treatment techniques such as aerobic granular sludge might be considered appropriate. Recent studies showed that an implementation of fungi in suspended granular biofilms usually occurs in sequencing batch reactors (SBRs) (Beun et al., 1999; Etterer and Wilderer, 2001).

The present study investigates the possible structural involvement of fungi in granular biofilms. Since little is known about the identity of these organisms in granular biofilms, this study also examines the fungal diversity in two SBRs, operated with malthouse and artificial wastewater. The SBRs contained fungi initially in the flocculent and later in granular sludge. Experience from numerous studies on fungal diversity in natural habitats has shown that the identification of fungi is often very complex. Fungi comprise high species richness and show high micromorphological similarities. Additionally, they exhibit multiple life-cycle types and can often not be cultured using standard culturing techniques (Kowalchuk, 1999; Bornemann and Hartin, 2000; van Elsas et al., 2000). Consequently, studies on the diversity of fungi, within a broad taxonomic range, require the use of molecular methods such as 18S rRNA gene sequence and ITS analyses (Kowalchuk et al., 1999). This study applies 18S rRNA and 5.8S rRNA gene sequences and internal transcribed spacer (ITS) sequence analyses. Appropriate molecular methods for the identification of these organisms will be of interest for a broad research community. Here, method improvements such as DNA extraction for filamentous fungi from wastewater are included as well as the evaluation and preparation of appropriate primer sets to assess the diversity of fungi in the granules. Moreover, the phylogenetic markers used are evaluated.

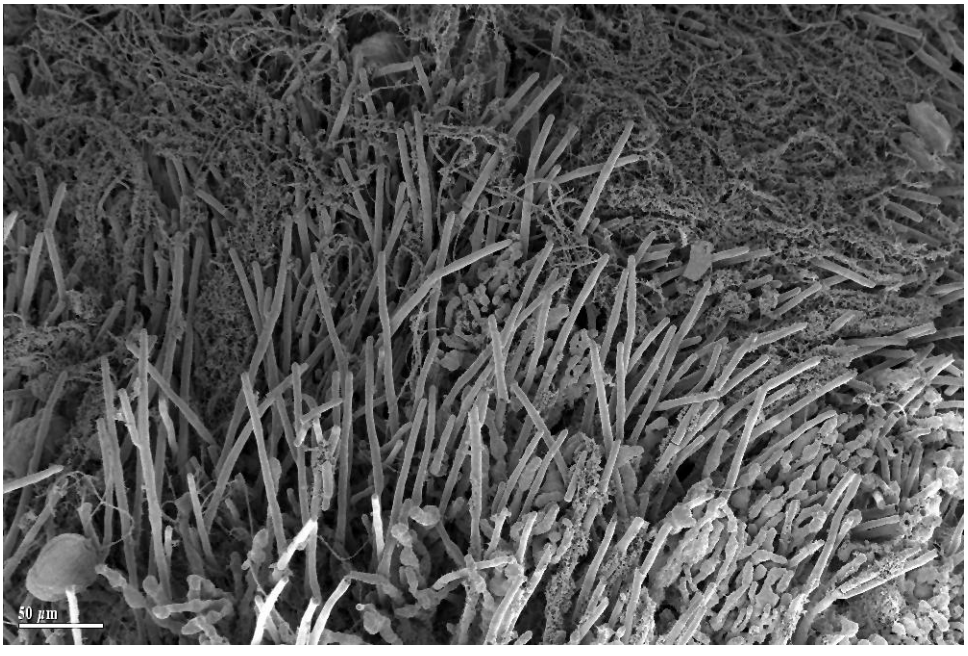


Fig. 4: Fungal hyphae protruding from the surface of a granule.
Scanning electron micrograph (by G. Wanner, S. Weber, 2007).

A.6 Motivation and aims of this study

Wastewater treatment is crucial nowadays as clean water becomes a scarce resource being vital for concurrently growing humankind. Consequently, the need for efficient and space- and energy-saving wastewater treatment techniques is driving research. As described above, aerobic sludge granules are regarded as one of the most effective and future-oriented technique in wastewater treatment. They incorporate diverse eukaryotic and prokaryotic microbes which have been proven as very efficient for wastewater purification.

This study investigates the microbial composition and structure of granules from different wastewaters. The diversity and role of fungi and protozoa in granules, which was neglected so far, is especially examined. It contributes significant tessera to fundamental and practical wastewater ecology research by addressing several questions:

- Which methods can be regarded as adequate for the monitoring of prokaryotes and eukaryotes in microbial granules?
- Are there different phases of granule formation?
- How are fungi and protozoa, i.e. ciliates, involved in the granule formation process?
- How are protozoan, fungal and bacterial community profiles changing during granule development and does wastewater composition also play a role?
- Which ciliates, bacteria and fungi entail the diversity in aerobic granules?
- How is the structure (matrix) of granules built up with regard to prokaryotic and eukaryotic organisms?
- Are there associations and interactions between protozoa, fungi and bacteria to be observed?

To address these questions, a combination of different microscopic and molecular methods was used.

B Material and Methods

All material and methods used in this study are described in detail in the original publications in Appendix A-C:

General topics as reactor set-up and operation, sampling frequency, sample fixation and preparation are described in Appendix A, B and C.

The improved FISH protocol and scanning electron microscopy are specified in Appendix A.

The identification of bacteria and ciliates is specifically described in Appendix C.

The identification of fungi, including DNA extraction, PCR and cloning, sequence analysis and phylogenetic analysis is specified in Appendix B.

C Results and Discussion

C.1 Elucidation of the granules structure

C 1.1 Granule formation in SBR setups with industrial wastewater

➲ Appendix A / C

In this study granule formation was observed within four SBR setups. They were operated with dairy, malthouse, brewery, and artificial wastewater. For the latter, two reactors with different air flow rates of 4 l min^{-1} and 6 l min^{-1} were used. The SBR with dairy wastewater was a pretest reactor where protozoan diversity was investigated and their possible function in the granule formation process was observed for the first time, as described below (C 1.2). This pretest led to the ideas and concept of this work.

Granules derived from the diverse wastewater types appeared different in size and habit (Fig. 4). Mature granules from malthouse wastewater were always completely brown colored (Fig. 5 B, E, I). Granules from dairy and synthetic wastewater appeared mostly white to beige (Fig. 5 A, C, D, G, H) and only sometimes dark in the center (Fig. 5 F). High substrate concentrations in the SBR with brewery wastewater gave rise to large granules of 5 to 6 mm diameter on average. This phenomenon of granules enlargement was also reported before for anaerobic granules (Grotenhuis et al., 1991). Brewery granules were dominated by filamentous bacteria and exhibited no satisfying settling capabilities as granules from all other SBRs did.

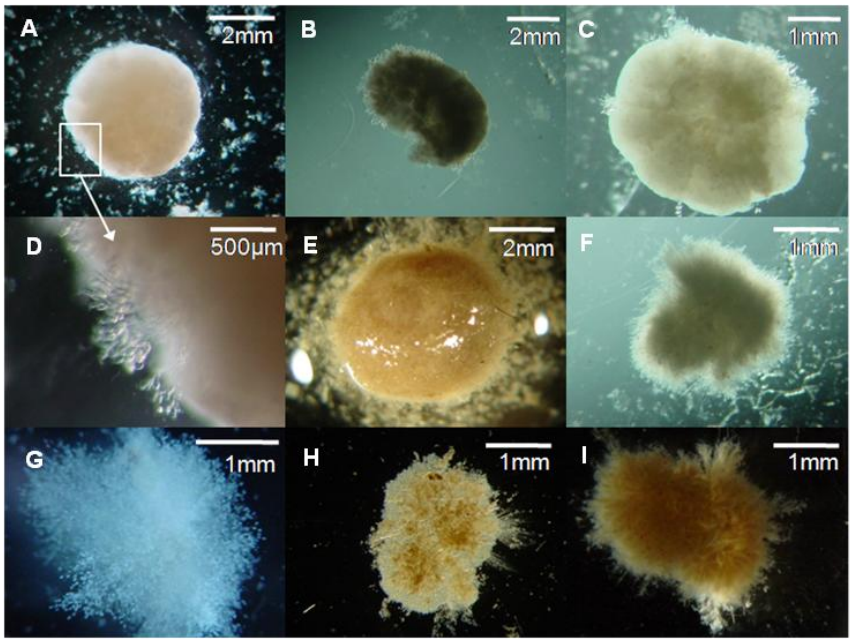


Fig. 5: Granules from different wastewater types (malthouse, synthetic, brewery) differ in size and habit. The pictures in the bottom row represent granular precursors - condensed sludge flocs with plentiful ciliate colonies.

C 1.2 Role of ciliates and fungi in the granule formation process

➲ Appendix A

A fundamental question to be solved was to what extent ciliated protists, i.e. of the subclass *Peritrichia*, and fungi are involved in the structural development of aerobic sludge granules. This was not investigated in studies on granular biofilms before. However, as mentioned above (A. 3), the structure of granules is crucial for their functionality and settling properties which influence the clarification performance of a SBR. As mentioned above protozoa and fungi are also desirable organisms in granular sludge since they generally comprise many advantageous characteristics for the purification efficacy of wastewater (see A.3) The combination of scanning electron microscopy SEM, light microscopy and confocal laser scanning microscopy CLSM, together with a modified FISH protocol as used in this study, provides a powerful set of tools to explore the structure of microbial granules.

This study proves that ciliate stalks and fungal hyphae can support the development of granular sludge by serving as a "backbone" within the biofilms, i.e. a substratum for bacteria to grow on. As revealed by microscopy and illustrated in Appendix A, Fig. 3 (p. 6235), it was shown for four different SBR setups with dairy, malthouse, brewery, and synthetic wastewater, that in presence of ciliates of the taxon *Peritrichia* granular development takes place in three phases:

In phase 1, swarming ciliates settle on activated sludge flocs often building tree-like colonies.

In phase 2, a massive growth of ciliate cells is observed. The flocs condense while EPS producing bacteria settle on ciliate stalks and stalk remnants. These condensed aggregates are considered to be granule precursors.

In phase 3, the cell bodies of the ciliates are likewise colonized by bacteria, embedded in the expanding biofilm and finally overgrown (Appendix A, Fig. 5, p. 6236). Ciliates either die during that process or build swarming cells to escape and settle again on other granule surfaces. Thus, granular biofilms might grow until they reach a steady-state size due to abrasion and washout (Batstone et al., 2004).

For fungi, a similar process as described above was observed: The filaments serve as backbone, however, they do not lyse during phase 2 and 3. Hyphae germinate once in the activated sludge flocs, grow fast and continuously, and are never completely overgrown by bacteria. The filament tips, i.e. the active growing region, are only sparsely colonized with bacteria (Appendix A, Fig. 7, p. 6237). Dense core zones and, later, compact granules with several protruding fungal filaments are found to develop.

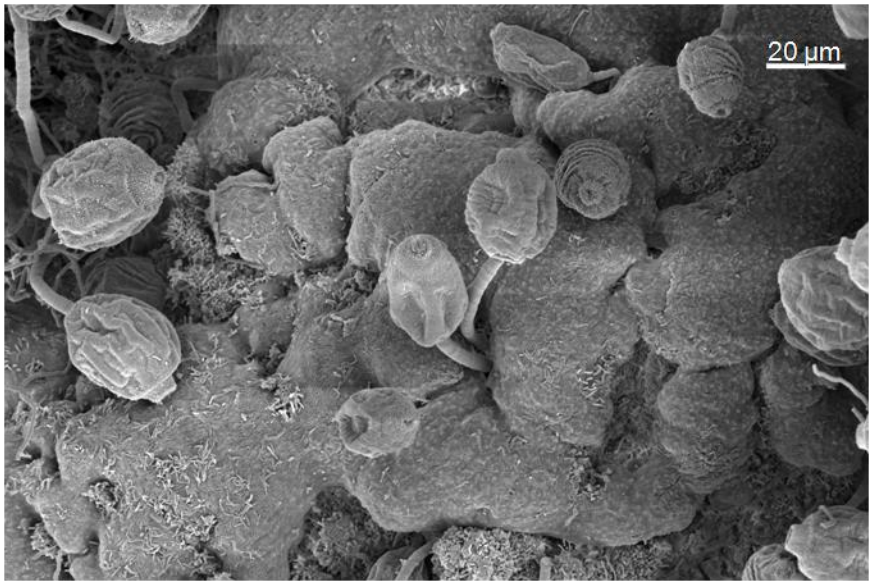


Fig. 6: Ciliate cells on the granules surface, partially embedded in the bacteria-EPS matrix

A further observation made in this study was, that fungi and ciliates appeared very seldom to grow together in the same granule. This was only the case in the setup with synthetic wastewater. Normally, granules comprise a community either with fungi or with ciliates.

C 1.3 Layered structure of granules

➲ Appendix A / C

Granules may develop several microbial layers as it has been reported in other case studies on anaerobic and aerobic granular sludge (Ahn et al., 2002; de Kreuk et al., 2005; McSwain et al. 2005). The layers were sometimes composed of distinct bacterial species with various functional tasks (Tay et al., 2003; Aoi et al., 2004; Batstone et al., 2004; Tsuneda et al., 2004; Abreu et al., 2007). In this study, different structured zones were found as well. Mature granules consisted of a dense core zone and a more loosely structured fringe zone (Appendix A, Fig. 5, p. 6237). The core zone comprised densely growing bacteria and EPS, whereas the fringe zone consisted mainly of bacteria and ciliate stalks or fungal hyphae. Bacteria appeared as manifold morphotypes in both layers (Appendix A., Fig. 9, p. 6238). In very compact mature granules, the fringe zone comprised ciliate stalks or hyphae only. The expansions of core and fringe zone differed from granule to granule, depending on wastewater type and developmental phase. Remnants of fungal hyphae or ciliate stalks were included in the outer parts of the core zone. No specific functional bacterial layers were found with FISH analysis (see Appendix C, 3.3).

C.2 A modified FISH protocol to investigate granular biofilm slices

➲ Appendix A

To investigate the bacterial microbial diversity in granules and especially the association of bacteria and ciliate stalks in more detail, a FISH protocol was modified and applied for cryosections of granules (Fig. 7). Bacteria, ciliates, and fungi could be detected simultaneously with this approach. Also cut ciliate cells could be investigated. The protocol is described in detail in Appendix A (pp. 6234-6235). With granules derived from malthouse and synthetic wastewater, all steps of the protocol worked without problems. However, the bulky brewery granules contained many polymers which acted like antifreeze agents. Therefore granules appeared sludgy and wet, even at -20°C. Thus, they could not always be properly sectioned with the cryotome. In this case, FISH was carried out with parts of ciliate colonies removed with tweezers from the surface of fixed granules to investigate the bacteria-ciliate associations. Examples for signals received after granule-FISH are shown in Appendix A, Fig. 1 and 2 (p. 6234).

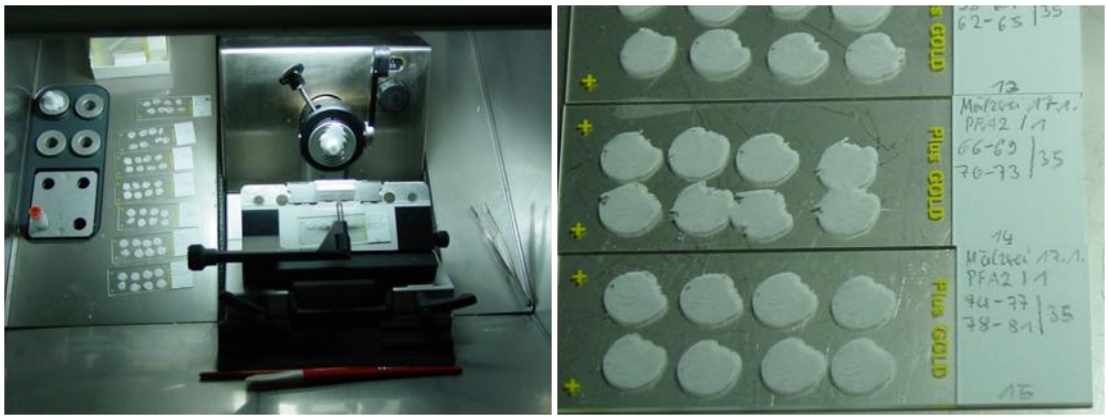


Fig. 7: Sectioning of granular biofilms. Granules were embedded after a fixation procedure in frozen section medium and cut into 25-30 µm slices with a cryotome (left). Slices placed on gelatine-coated slides shortly before heat fixation (right).

C.3 Protozoan diversity

➲ Appendix C

The diversity of ciliates was examined in all SBR reactors of this study. In dairy (D), malthouse (M), and brewery (B) wastewater a clear succession of ciliate communities was observed during the granule formation process. Ciliate populations always started with a mixture of sessile and free swimming organisms and ended up in the dominance of specific genera. All ciliate taxa present in the reactors in the different granule development phases are listed in Appendix C, Fig. 1A-C. The ciliate community in SBRD included mainly the genera *Vorticella*, *Carchesium*, *Epistylis*, *Opercularia*, *Litonotus*, *Prorodon*, and *Tetrahymena*. In SBRM and SBRB especially the genera *Vorticella*, *Opercularia*, *Epistylis*, *Epicarchesium*, *Litonotus*, *Acineria*, *Colpoda*, and *Aspidisca* were found. In SBRB *Tokophrya quadripartita* and species of the genus *Pelagothrix* were detected additionally, while *Colpoda* spp. was not found. Figure 2 in Appendix C represents a selection of peritrichous ciliates found in the diverse granules. Some ciliate species were only temporarily detected maybe due to their ability to form dormancy cysts or because they died or were washed out. After several weeks, the ciliate communities in SBRD, SBRM, and SBRB were dominated by specific genera of ciliates including *Opercularia* spp., e.g *Opercularia asymmetrica*, *Tetrahymena* sp., *Epistylis chrysemydis*, *Epistylis entzii*, *Vorticella* spp., and specifically in SBRD representatives of the *Vorticella microstoma*-complex. The ciliate communities in the synthetic wastewater-fed steady state reactors SBRS1 and SBRS2 were dominated solely by *Opercularia* spp. over the whole observation period of 4 months.

The observation that specific protozoa dominated the community after granule formation led to the conclusion that some ciliate organisms, especially *Opercularia* spp. and *Epistylis* spp., which occurred in all reactors on mature granules, proved to be very flexible in coping successfully with different types of wastewater.

C.4 Fungal diversity

In malthouse and synthetic wastewater, fungi were found both in the granules and also before granulation. Since hardly anything is known about fungi in aerobic granules, their diversity in the samples was examined by sequence analysis of the 18S rRNA and 5.8S rRNA genes and the conterminal ITS regions. The use of molecular methods to assess fungal diversity is necessary due to difficulties in culturing and micromorphological differentiation (Kowalchuk, 1999). In the field of phylogeny the rRNA gene operon analysis is a common and well established method for many eukaryotes (White et al., 1990; Aleshin et al., 1998; Smit et al., 1999; Borneman and Hartin, 2000; Fried, 2002; Hughes and Piontkivska, 2003; Schaap et al., 2006). Following recommendations of other studies (Chen et al., 2001; Manter and Vivanco, 2007) isolates were additionally examined by analysis of the highly variable transcribed spacer regions ITS 1 and ITS 2 to probably receive a higher phylogenetic resolution than with rRNA gene analysis alone.

C.4.1 Phylogenetic affiliation of the fungal wastewater clones

➲ Appendix B

The sequences of 41 isolates were analyzed and assigned to the taxonomic groups within the *Ascomycota* and *Basidiomycota*, listed in detail in Tab. 3, including EMBL sequence accession numbers, and shown in an overview in Appendix B, Fig. 2 (p. 249). Thereby most clones were assigned to the *Ascomycota*, and the fungal diversity in malthouse wastewater was clearly higher than in synthetic wastewater. Many fungi were found to be present in the samples before and after granulation. These organisms are of exceptional interest since they may particularly contribute to the biofilm structure of the granules. In malthouse wastewater *Claviceps* and relatives, *Tremellomycetes*, and *Pleosporaceae* were found both in flocculent and granular sludge; representatives of *Aureobasidium* and relatives were detected in flocculent sludge only. In granules from synthetic wastewater structure-supporting fungal filaments were isolated directly from single granules and identified by sequence analysis as representatives of *Aureobasidium* and relatives. These clones showed high sequence similarities to *A. pullulans*, which often occurs in activated sludge (Subramanian, 1983).

Clone	Taxonomic group	PCR-primer forward/ reverse	18S rRNA gene sequence length + ITS (bp)	Sequence accession number (EMBL)
malthouseww_clone16	*Pleosporaceae*	EF60f / ITS4r	2071	FM178242
malthouseww_clone18	*Pleosporaceae*	EF60f / ITS4r	2219	FM178244
malthouseww_clone20	*Pleosporaceae*	EF60f / ITS4r	2152	FM178246
malthouseww_clone22	*Pleosporaceae*	EF60f / ITS4r	2255	FM178248
malthouseww_clone34	*Pleosporaceae*	EF60f / ITS4r	2429	FM178260
malthouseww_clone35	*Pleosporaceae*	EF60f / ITS4r	2519	FM178261
malthouseww_clone27	*Pleosporaceae*	EF60f / ITS4r	2598	FM178253
malthouseww_clone15	*Xylariales*	EF60f / ITS4r	2253	FM178241
malthouseww_clone23	*Candida boleticola*	EF60f / ITS4r	1828	FM178249
malthouseww_clone17	*Claviceps et rel.*	EF60f / ITS4r	2306	FM178243
malthouseww_clone19	*Claviceps et rel.*	EF60f / ITS4r	2274	FM178245
malthouseww_clone24	*Claviceps et rel.*	EF60f / ITS4r	2136	FM178250
malthouseww_clone25	*Claviceps et rel.*	EF60f / ITS4r	2022	FM178251
malthouseww_clone26	*Claviceps et rel.*	EF60f / ITS4r	2021	FM178252
malthouseww_clone21	*Claviceps et rel.*	EF60f / ITS4r	2110	FM178247
malthouseww_clone10	*Claviceps et rel.*	EF4f / ITS4r	2160	FM178236
malthouseww_clone32	*Claviceps et rel.*	EF4f / ITS4r	2158	FM178258
malthouseww_clone8	*Tremellomycetes*	EF4f / ITS4r	2004	FM178234
malthouseww_clone9	*Tremellomycetes*	EF4f / ITS4r	2101	FM178235
malthouseww_clone12	*Tremellomycetes*	EF4f / ITS4r	1827	FM178238
malthouseww_clone14	*Tremellomycetes*	EF4f / ITS4r	1743	FM178240
malthouseww_clone28	*Tremellomycetes*	EF4f / ITS4r	2123	FM178254
malthouseww_clone29	*Tremellomycetes*	EF4f / ITS4r	2014	FM178255
malthouseww_clone30	*Tremellomycetes*	EF4f / ITS4r	2229	FM178256
malthouseww_clone31	*Tremellomycetes*	EF4f / ITS4r	2114	FM178257
malthouseww_clone33	*Tremellomycetes*	EF4f / ITS4r	2109	FM178259
malthouseww_clone7	*Tremellomycetes*	EF4f / ITS4r	2102	FM178233
artificialww_clone38	*Tremellomycetes*	EF4f / ITS4r	1678	FM178264
malthouseww_clone11	*Aureobasidium et rel.*	EF4f / ITS4r	2161	FM178237
malthouseww_clone13	*Aureobasidium et rel.*	EF4f / ITS4r	2167	FM178239
artificialww_clone36	*Aureobasidium et rel.*	EF4f / ITS4r	2162	FM178262
artificialww_clone41	*Aureobasidium et rel.*	EF4f / ITS4r	2074	FM178267
malthouseww_clone5	*Thelebolaceae*	EF4f / ITS4r	2137	FM178231
malthouseww_clone1	Unidentified fungus	EF4f / ITS4r	1992	FM178227
malthouseww_clone2	Unidentified fungus	EF4f / ITS4r	2204	FM178228
malthouseww_clone3	Unidentified fungus	EF4f / ITS4r	1991	FM178229
malthouseww_clone4	Unidentified fungus	EF4f / ITS4r	1995	FM178230
malthouseww_clone6	Unidentified fungus	EF4f / ITS4r	2004	FM178232
artificialww_clone37	Unidentified fungus	EF4f / ITS4r	1620	FM178263
artificialww_clone39	Unidentified fungus	EF4f / ITS4r	1991	FM178265
artificialww_clone40	Unidentified fungus	EF4f / ITS4r	2004	FM178266

Tab. 3: Overview of all 18S rRNA gene sequence/ITS clones received after sequence analysis during this study. Clone names include sequencing batch reactor type (SBR - operated with malthouse or artificial wastewater) from which samples were derived. The phylogenetic affiliation of the clones to different taxonomic groups within the fungi after ARB analysis, used PCR primer combinations, and length of the clone sequences in basepairs (bp) are shown.

C.4.2 Methodological findings and improvements

C.4.2.1 DNA extraction protocol and primer selection

Three different DNA extraction methods were tested as described in Appendix B (pp. 248). The protocol including a combination of enzymatic and mechanical lysis of fungal filaments is recommended as most adequate technique for the extraction of fungal DNA from wastewater. For the following PCR adequate primers were tested which should fulfill three requirements: (1) they should cover the phylogenetic spectrum in the database as far as possible, (2) discriminate against other eukaryotes, especially ciliates, and, due to their target sites, and (3) allow amplification of the complete fungal 18S rRNA gene and the conterminal ITS region. Only very few primers were found after intensive literature research to fulfill these three prerequesites (see Appendix B, p.252). Following the recommendation of Martin and Rygiewicz (2005) to use multiple primer sets for the identification of fungi in natural habitats, two primer pairs, EF60f/ITS4r and EF4f/ITS4r, were used for the amplification of fungal DNA from the SBR sludges analyzed in this study. EF60f was designed within this study whereas the other primers EF4f and ITS4r have already been published (Appendix B, p.248).

C.4.2.2 Phylogenetic analysis

18S rRNA gene analysis allowed a phylogenetic differentiation down to levels of families or genera. The phylogenetic assignment of the 18S rRNA gene sequences to taxonomic groups was always consistent with the assignment of the associated ITS sequences within these taxa. In one case a higher discriminatory power was proven for the ITS sequences. The ITS tree of *Claviceps* and relatives (Appendix B, Fig. 4, p.251) contains three clone sequences, i.e. clones 21, 32, and 10, representing organisms of a monophyletic group with *Tolypocladium* spp. The sister group status of *Tolypocladium* and these three sequences was significantly supported in the ITS tree, but could not be resolved in the 18S rRNA gene sequence

phylogeny. However, for all other ITS clone sequences in this study, no further refinement compared with the 18S rRNA gene trees was possible. The reason is the number of publicly available ITS reference sequences for the respective groups which is still too small. Regarding the present substantial sequencing advances in fungal biology there is an indispensable need to expand this dataset, especially for complex environmental samples. This study supports this aim, providing 41 new full sequences of the ITS region (sequence accession numbers are listed in Tab. 3, sequences published at the EMBL database http://www.ebi.ac.uk/embl in association with original article in Appendix B).

C.5 Bacterial diversity

➲ Appendix A/C

Bacteria represented in terms of biomass the main microbial components of granules. They were embedded mostly in clusters and sometimes as single cells in the EPS matrix. Microscopic analyses revealed that bacteria occurred in a wide morphological variety. Rods, cocci, filaments and tetrad-arranged individuals were found, and occasionally some spirilla. Sometimes all forms occurred in the same granule. The micrographs in Figure 9, Appendix A (p. 6238) illustrate different bacterial morphologies.

FISH analyses disclosed the high diversity of bacteria in all SBR sludges. The approach with 46 *Prokarya*-specific rRNA targeted probes showed differences in community composition for the diverse wastewaters and granulation phases (see Tab. 1, Appendix C). Some taxonomic groups could only be found in phase 1 or phase 2 within an individual SBR setup, e.g. in SBRB, *Firmicutes* were found in granules only and *Actinobacteria* solely in flocculent sludge. Overall, the taxa *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Actinobacteria* prevailed.
Most of the bacteria were rod-shaped, a form which is advantageous in biofilms to facilitate cell-to-cell connection and to diminish the impact of fluid shear (Young, 2006). Filamentous bacteria were present in large numbers in SBRB and identified as *Sphaerotilus natans*, *Thiothrix* sp. and *Thiothrix nivea*.

Within the *Alphaproteobacteria*, which are morphologically extremely diverse (Dworkin and Falkow, 2006) no genera could be specified with the rRNA targeted probes selected in this study. However, the tetrad-arranged organisms might represent glycogen-accumulating

organisms (GAOs) as they share morphology with *Defluviicoccus* sp., a genus being reported as a common GAO in other recent studies (Wong et al., 2004, 2007; Oehmen et al., 2006).

Within the *Betaproteobacteria* representatives of the *Nitrosomonas oligotropha* lineage, which are common representatives of the ammonia oxidizers (AOB) in wastewater purification as well as nitrite oxidizing (NOB) *Nitrospira* sp. were detected. The latter were rod-shaped and arranged in chains or spherical clusters, depending on wastewater type. In SBRB, the filamentous bacterium *Sphaerotilus natans* was additionally identified, which is readily being found in food industry and brewery wastewater (Lemmer and Lind, 2000).

For the *Gammaproteobacteria,* no specific genera could be identified with the rRNA targeted probes selected in the present study except for the filamentous sulfur bacterium *Thiothrix nivea* being numerously present in SBRB.

C.6 Mutual impact of protozoa and bacteria

➲ Appendix C

C.6.1 Spatial distribution of ciliates and bacteria in the granula biofilms

In general, a bilayered structure as described in C.1.3 comprising a core zone (bacteria) and a fringe zone (ciliates) was observed in the granules. Taking a closer look at the bacterial layer formation by FISH analysis, no spatial preferences of specific bacterial taxa in the biofilm were observed as it was reported in some other studies (Tay et al., 2003; Batstone et al., 2004, Abreu et al., 2007). The detected taxa were mostly arranged in clusters and these were homogenously allocated over the whole granule. A similar case was described by Wang et al. (2004). The nearly homogenous distribution of bacteria in this study might be due to a balanced nutrient supply throughout the biofilm by virtue of channels, voids, and pores which are typical for most aerobic granules (Tay et al., 2003, Wang et al., 2004).

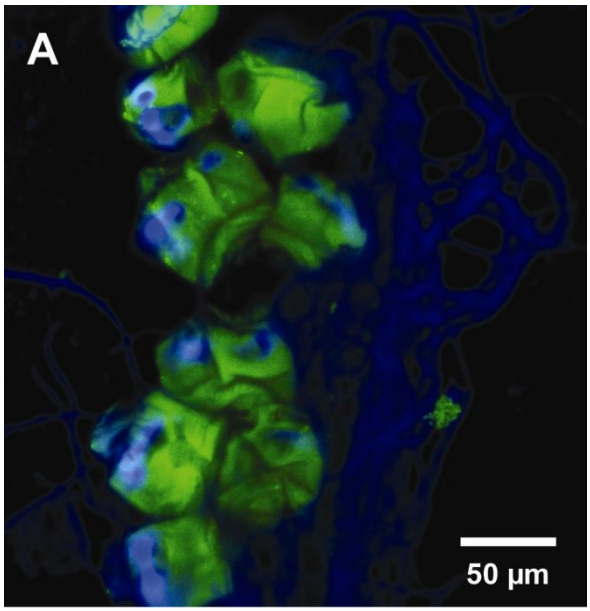
C.6.2 Association of ciliates and bacteria

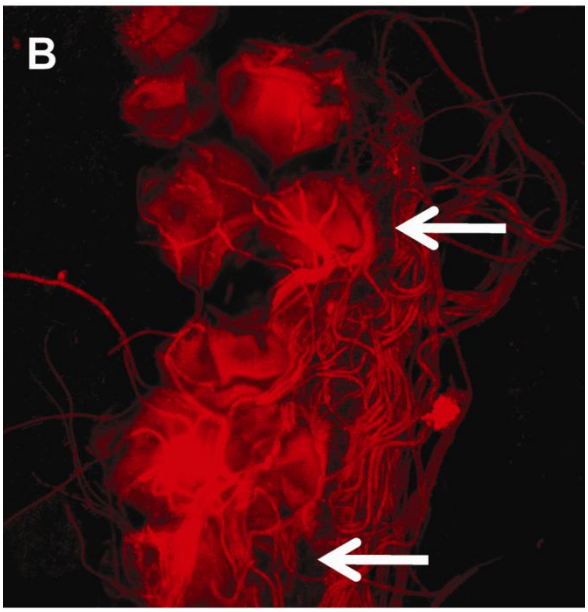
Bacteria were associated with ciliate stalks in large numbers. In micrographs (see Fig. 5, Appendix C) mostly rod-shaped cells were detected abutting lengthwise along the stalks or grabbing hold orthogonally to the stalks with their pili. The question if bacteria-ciliate associations are at random or a matter of species- or morphology-based relationship should be

answered by FISH experiments. FISH analysis carried out with all rRNA targeted probes which were already tested positive in this study showed that mainly *Gammaproteo*- and *Betaproteobacteria*, and within the latter *Nitrospira* sp. were associated with the ciliate stalks. In SBRB sludges also *Actinobacteria* were detected on the stalks. However, no other distinctive bacteria were found associated with the ciliates. All bacteria detected within these FISH approaches to be attached to the stalks were rods. Thus, this might be an additional indication for the observations of Young (2006) that cell shape seems to be more decisive for attachment than the specificity of different bacterial taxa.

C.6.3 Competition between ciliates and bacteria

Biofilm formation is a strategy of microorganisms to protect themselves against harmful environmental influences. However, within the biofilms a struggle for survival and individual vantages exists. In the SBRB granules filamentous bacteria and peritrichous ciliates were observed to compete for settling sites and space to grow on the granules due to their similar growth pattern with both protruding from the granule surface. Sometimes fast growing filamentous bacteria enwrapped ciliates and hampered their cilia beat, resulting in the breakup of ciliates (Fig. 6). The formation of bacterial filaments may have different causes with respect to environmental conditions. Avoiding ciliate predation might be one reason (Shikano et al., 1991; Matz et al., 2006). Single, non-filamented bacteria cells detached from granular biofilms are the main food supply for ciliates, which ingest them together with organic particles. A FISH survey on the content of ciliate food vacuoles in this study showed no preferences for the examined ciliate species to ingest specific bacterial taxa. Most of the bacteria found in the granules were also detected in the vacuoles. Solely *Actinobacteria* were never found in vacuoles. This might be due to the stodgy cell wall of gram-positive bacteria (Gonzalez et al., 1990; Iriberri et al., 1994, Pernthaler J., 2005).





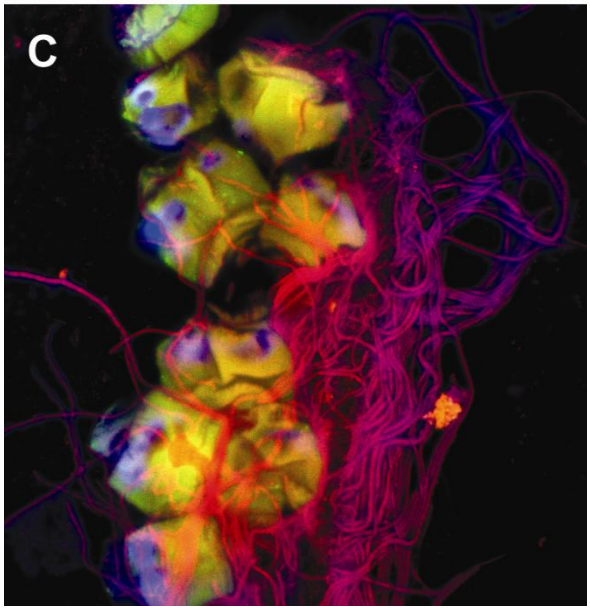


Fig. 8: Probe-related FISH signals on a tree-like ciliate colony from the surface of a single granule developed in malthouse wastewater. The images are recorded by CLSM after FISH with the *Bacteria*-specific mix of the EUB338 probes (Cy3-labeled; red), the *Eukarya*-specific probe EUK516 (fluorescein-labeled; green) and DAPI staining (blue). They show protist cell bodies surrounded partially by filamentous bacteria which are attached to the ciliate stalks. Overlays of probe EUK516-Fluos signal and DAPI staining (A) and of probe EUK516-Fluos signal, probes EUB338-Cy3 signals, and DAPI staining (C); Cy3 signal of EUB338 probes and slight autofluorescence of ciliate cells (B). White arrows indicate the location of ciliate stalks, since non-contractile stalks are lacking rRNA and therefore do not constitute a probe target.

C.7 Conclusions and outlook

In the present study, the structure and microbial composition of aerobic activated sludge granules originating from five differently operated sequencing batch reactors were described. Therein the diversity of bacteria, fungi, and protozoa within the sludges was determined. In addition associations and possible interactions of prokaryotes and eukaryotes were elucidated. The following conclusions can be drawn from the obtained results:

- The combination of SEM, light microscopy and CLSM, together with a modified FISH protocol, provides a powerful set of tools to explore and monitor the structural development of microbial granules and is thus recommended for future biofilm studies.
- Population dynamics of ciliates obviously follows a similar pattern during granular biofilm formation within all the setups tested: A high diversity of ciliates in the flocculent seed sludge is always followed by establishment of specific peritrichous ciliate taxa during granule formation.
- The diversity of ciliates is expectedly shown to be influenced by a variety of selection factors such as operation parameters or wastewater composition.
- The selection and succession of peritrichous ciliate species might be influenced by the presence of bacterial filaments constituting strong competitors for settling sites on the biofilm surface.
- Bacteria always use the stalks of ciliates as substratum to grow on. The physical shape of bacteria might thereby play an important role with rods seemingly being the preferred shape.
- Analyses in this study based on whole cell FISH with rRNA targeted probes support the shape-based relationship hypothesis, since associated bacteria were exclusively rod-shaped.
- Bacterial diversity in granules seems to depend on wastewater composition. However, in terms of spatial arrangement the bacterial taxa within the biofilm are shown to be homogenously and randomly distributed.
- To assess the diversity of fungi and their possible role in granular SBR biofilms, an improvement of the required molecular techniques was necessary. The combined approach of DNA extraction, application of adequate primer sets for 18S rRNA genes and ITS amplification of fungi was proved successful.
- It was shown that some of the identified fungi might be involved in the structural buildup of granular sewage biofilms because they could be found before and after granulation being finally incorporated in the granules.

- Regarding the common knowledge of the metabolic potential of fungi and the high fungal diversity presented in this study, the role, diversity, function, and application of fungi in activated sludge processes should be further examined.
- The further specific development of technical procedures to integrate fungal filaments in compact granular sludge will support the purposeful use of fungi in wastewater treatment.

D Summary

D.1 Summary

Present-day biological wastewater purification on a highly effective level is crucial in a world with very fast growing mankind. Thus, it is indispensable to optimize processes of promising treatment techniques as the use of granular sludge and additionally understand the complex composition, structure, and microbial population dynamics of such biofilms.

This study investigated the structure and microbial community composition of aerobic sewage granules from sequencing batch reactors (SBR) which were each maintained over several months with different industrial wastewater from malthouse, dairy, and brewery as well as with a synthetic mixture. The diversity and role of stalked ciliates of the subclass *Peritrichia* and of fungi during granule development phases was monitored using microscopic and molecular methods. Thereby it was shown that these eukaryotes are exceedingly responsible for the structural development of activated sludge flocs to granular biofilms: Ciliate stalks and fungal hyphae constitute a substratum for bacteria to grow on. The present study also indicated that the diversity of *Peritrichia* was always high in aggregating flocs but ended up in the dominance of only some genera on the surface of mature granules, i.e. *Opercularia, Epistylis,* and *Vorticella*. This implies that these ciliates adapt best to the granular sludge environment and are eminently dedicated to support granular biofilm structure. Within the fungi some genera were identified by rDNA sequencing and phylogenetic examinations before and after granule development. Thus, these fungal taxa are assumed to particularly constitute the hyphae network which supports the overall biofilm structure. The 41 full sequences of fungal 18S rDNA, 5.8 rDNA, and adjacent ITS 1 and 2 regions obtained thereby strongly enlarge the existing public dataset of fungal sequences from wastewater environment. Since scarcely anything is known about general fungal diversity in activated sludge from industrial wastewater the newly acquired sequence data together with their phylogenetic evaluation and improved DNA extraction methods for wastewater fungi highly support and enrich future research thereon.

Moreover, bacterial diversity was monitored in this study for all wastewater types before and after granulation. Both the overall bacterial composition and the identity of specific bacteria attached to ciliate stalks and fungal hyphae were examined. For the latter a FISH protocol for the analysis of granule slices was developed during this study to monitor the bacteria-eukaryote associations within the developing biofilms. Results showed no spatial preferences of bacteria in the whole granule and no preferences of distinct bacterial taxa attached to

ciliates or fungi. Bacterial taxa were evenly distributed in the granules and associations with eukaryotes were recognized to be more a matter of shape than of taxa. Only rods affiliated to diverse bacterial taxa were sticking to stalks and hyphae. This finding was supported by scanning electron micrographs (SEM) of the granule samples. SEM and FISH examinations also elucidated the structure of mature granules which were found to throughout consist of a compact core zone and a loosely structured fringe zone.

Summarized, the present work represents a comprehensive contribution to the understanding of complex wastewater biofilm composition, structure and microorganism dynamics with focus on the interactions of prokaryotes and eukaryotes.

D.2 Zusammenfassung

Effektive Abwasserreinigung zur Erhaltung der Lebensgrundlage „Wasser“, ist in der heutigen Welt mit einer rasch steigenden Bevölkerungsdichte wichtiger denn je. Es bedarf sowohl der ständigen Weiterentwicklung und Optimierung bestehender biologischer Reinigungstechniken, wie beispielsweise des granulären Belebtschlamms, als auch einer Erweiterung des Verständnisses über den strukturellen Aufbau von komplexen mikrobiellen Biofilmen.

Die vorliegende Studie untersuchte Struktur und mikrobielle Zusamensetzung aerober Belebtschlammgranula aus Sequencing-Batch-Reaktoren (SBRs). Die SBRs wurden mit verschiedenen Abwässern aus jweils einer Mälzerei, Molkerei und Brauerei, sowie mit einer künstlich zusammengesetzten Abwassermischung, betrieben. Die Diversität und Rolle von gestielten Ciliaten der Unterklasse *Peritrichia* und von Pilzhyphen während der Bildungsphase der Granula wurde mit Hilfe mikroskopischer und molekularer Methoden untersucht. Dabei zeigte sich, dass sowohl Ciliaten als auch Pilze deutlich an der Entwicklung von der Belebtschlammflocke hin zum granulären Biofilm beteiligt waren. Ciliatenstiele und Pilzhyphen stellten für Bakterien einen idealen Untergund dar, auf dem sie sich ansiedeln und voluminöse Biofilme bilden konnten. Die Diversität der *Peritrichia* in aggregierten Flocken war dabei stets sehr hoch, nahm jedoch im Laufe der Granulabildung ab, bis nur noch einige wenige Gattungen auf der Oberfläche reifer Granula siedelten. Hierbei handelte es sich vor allem um *Opercularia*, *Epistylis,* und *Vorticella*. Diese Beobachtung impliziert, dass diese Ciliaten sich besonders gut an die Umweltbedingungen auf den granulären Biofilmen anpassen und dadurch eine eminente Rolle bei der Strukturbildung übernehmen konnten.
Von den mittels rDNS Sequenzierung und nachfolgender phylogenetischer Analyse identifizierten Pilzgattungen konnten einige Taxa vor und einige auch noch nach der Granulierung nachgewiesen werden. Es wird demzufolge angenommen, dass diese durchweg präsenten Pilztaxa im Speziellen an der Biofilmstruktur beteiligt waren. Die erhaltenen 41 Vollsequenzen bestehend aus 18S rDNS, 5.8S rDNS und zugehörigen ITS 1 und 2 Regionen, erweitern den bislang existierenden und öffentlich zugänglichen Datensatz für Pilzsequenzen aus dem Habitat Abwasser signifikant. Da bislang nur wenig über die Diversität von Pilzen im Belebtschlamm industrieller Abwässer bekannt ist, tragen die im Rahmen dieser Arbeit optimierten DNS-Extraktionsmethoden für Abwasserpilze, als auch die ermittelten rDNS Sequenzen und deren phylogentische Evaluierung in hohem Maße zur weiteren Forschung auf diesem Gebiet bei.

Die bakterielle Diversität wurde für die untersuchten Abwassertypen ebenfalls vor und nach der Granulierung ermittelt. Es wurden jeweils die gesamte bakterielle Zusammensetzung der Proben, sowie die Identität spezifischer, an Ciliatenstielen und Pilzhyphen angehefteter Bakterien untersucht. Dabei wurde ein FISH-Protokoll zur Analyse von Granuladünnschnitten entwickelt, um die Bakterien-Eukaryoten Vergesellschaftungen in den enstehenden Granula zu monitorieren. Es konnten keine Präferenzen bestimmter Bakterien hinsichtlich der räumlichen Verteilung in den Granula festgestellt werden. Sämtliche identifizierte Bakterientaxa lagen innerhalb der Granula gleichmässig verteilt vor. Gleiches galt für den Verband von Bakterien mit Ciliatenstielen oder Pilzhyphen. Diese Vergesellschaftungen wiesen auf eine strukturell-funktionelle Natur hin, da nur Stäbchenbakterien, begünstigt durch ihre Form, mit Ciliatenstielen und Pilzhyphen assoziiert waren. Dies konnte auch in elektronenmikroskopischen Aufnahmen nachgewiesen werden. Elektronenmikroskopie und FISH trugen weiterhin zur Aufklärung der übergeordneten Struktur reifer, granulärer Biofilme bei. Diese bestanden stets aus einer kompakten Kernzone und einer locker strukturierten Randzone.

Zusammengefasst stellt die vorliegende Arbeit einen umfassenen Beitrag zum Verständnis komplexer Abwasserbiofilme bezüglich deren Struktur, Zusammensetzung und mikrobieller Dynamik im Hinblick auf Prokaryoten-Eukaryoten-Interaktionen dar.

E References

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F Appendices

Appendix A

Microbial composition and structure of aerobic granular sewage biofilms

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Microbial Composition and Structure of Aerobic Granular Sewage Biofilms[▽]

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Aerobic activated sludge granules are dense, spherical biofilms which can strongly improve purification efficiency and sludge settling in wastewater treatment processes. In this study, the structure and development of different granule types were analyzed. Biofilm samples originated from lab-scale sequencing batch reactors which were operated with malthouse, brewery, and artificial wastewater. Scanning electron microscopy, light microscopy, and confocal laser scanning microscopy together with fluorescence in situ hybridization (FISH) allowed insights into the structure of these biofilms. Microscopic observation revealed that granules consist of bacteria, extracellular polymeric substances (EPS), protozoa and, in some cases, fungi. The biofilm development, starting from an activated sludge floc up to a mature granule, follows three phases. During phase 1, stalked ciliated protozoa of the subclass Peritrichia, e.g., *Epistylis* spp., settle on activated sludge flocs and build tree-like colonies. The stalks are subsequently colonized by bacteria. During phase 2, the ciliates become completely overgrown by bacteria and die. Thereby, the cellular remnants of ciliates act like a backbone for granule formation. During phase 3, smooth, compact granules are formed which serve as a new substratum for unstalked ciliate swarmers settling on granule surfaces. These mature granules comprise a dense core zone containing bacterial cells and EPS and a loosely structured fringe zone consisting of either ciliates and bacteria or fungi and bacteria. Since granules can grow to a size of up to several millimeters in diameter, we developed and applied a modified FISH protocol for the study of cryosectioned biofilms. This protocol allows the simultaneous detection of bacteria, ciliates, and fungi in and on granules.

During the last 20 years, intensive research in the field of biological wastewater treatment and other applications has demonstrated that biofilms are often more efficient for water purification than suspended activated sludge. Today, the application of anaerobic and aerobic granular sludge in wastewater treatment is regarded as one of the most useful and promising biotechnologies. Granular sludge was described first for strictly anaerobic systems (26). In the late 1990s, the formation and application of aerobic granules was reported (30). Such granules are spherical compact aggregates of microorganisms, mainly bacteria, and extracellular polymeric substances (EPS). Granules can be described as "biofilm in suspension" and are considered to be a special case of biofilm formation, composed of self-immobilized cells (9, 16, 20). The application of granules for wastewater treatment shows many advantages. An outstanding feature is the excellent settleability (high settling velocity), which is a prerequisite to handle high liquid flows. Moreover, granular sludge provides high and stable rates of metabolism, resilience to shocks and toxins due to protection by a matrix of EPS (40), long biomass residence times, biomass immobilization inside the aggregates and, therefore, the possibility for bioaugmentation. Bioaugmentation can be regarded as an effective tool in the removal process of xenobiotica from wastewater (6, 41). Metabolic activities in the operating system can be kept at a high level because of the syntrophic associations which occur due to optimum distances between microbial partners at appropriate substrate levels (7). Various investigations of general characteristics of sludge granules, such as size, structure, settling performance, stability against shear forces, EPS content, reactor performance, and metabolism rates, were performed previously (13, 17, 24, 25, 28, 32). Spontaneous aerobic granulation of suspended aggregates is a phenomenon that has been most frequently observed in systems applying the sequencing batch reactor (SBR) concept. Theories about the crucial factors of granule development have been intensively discussed (39). However, in all studies of aerobic granules, the possible role of protozoa and fungi in the biofilm-forming process and the accompanying interaction with bacteria have, so far, been neglected. Several studies on fungi and protozoa in activated sludge systems in general demonstrated that these eukaryotic organisms fulfill a wide variety of important tasks in the biomass conversion and water clarification processes (10, 19, 21, 33, 35). It is known that protozoa or fungi are involved in the formation, structure and function of biofilms for several biofilm systems besides wastewater treatment (22). In preliminary observations of granular biofilms, we found stalked ciliates and, in some cases, fungi to be present in high numbers. The tree-like structure of ciliate colonies and the network of fungal filaments provided a distinctive enlargement of the area available for bacterial colonization. For that reason, this study focuses on the role that ciliates and fungi play in the structural formation of microbial granules derived from activated sludge. The combination of scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) with an adapted technique for fluorescence in situ hybridization (FISH) offers a powerful tool to visualize the detailed architecture and microbial composition of these granules.

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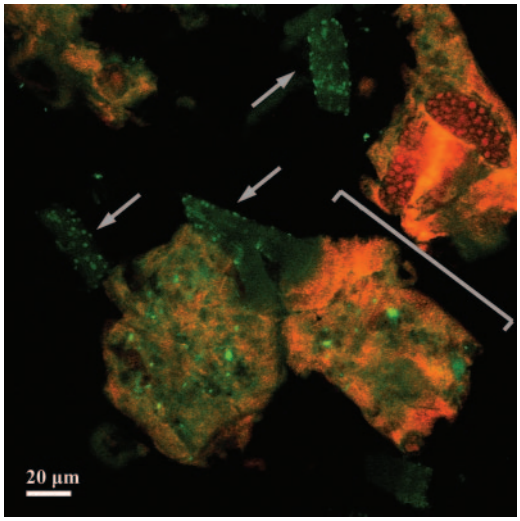

FIG. 1. Probe-related signals after FISH applied on a single-granule microsection of 30-μm thickness. The granule was developed in malthouse-derived wastewater. Images are recorded by CLSM after FISH with the *Bacteria*-specific mix of the EUB338 probes (fluorescein-labeled; green) and the *Eukarya*-specific probe EUK516 (Cy3-labeled; red). One stack out of 36 stacks of 0.42-μm thickness (each) is shown. Arrows show the branched stalks of a colony of the peritrichous ciliate *Epistylis* sp. covered by bacteria. The bracket marks the truncated cell bodies (zooids) of the colony.

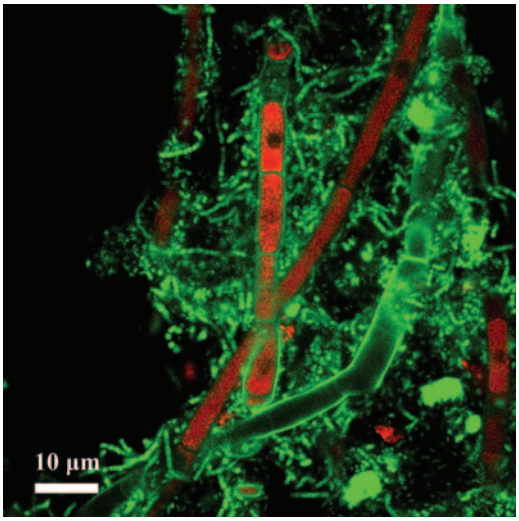

FIG. 2. Probe-related signals after FISH applied on a single-granule microsection of 30-μm thickness. The image shows fungal filaments colonized by bacteria in a granule developed in synthetic wastewater. Probe-related signals are recorded by CLSM after FISH with the *Bacteria*-specific mix of the EUB338 probes (fluorescein-labeled; green) and the *Eukarya*-specific probe EUK516.

MATERIALS AND METHODS

Reactor set-up and operation. Biomass was enriched in three lab-scale SBRs. Seed sludge was obtained from the municipal wastewater treatment plant in Garching, Germany. The SBRs were operated with different wastewater. SBR1 was fed with particulate-rich malthouse wastewater prepared by mixing barley dust with tap water as described elsewhere (39). SBR2 was operated with raw wastewater from a brewery (Bavarian State Brewery Weihenstephan, Freising, Germany) and SBR3 with synthetic wastewater according to previous recommendations (32). Different wastewater types and operational set-ups of the SBRs were chosen to investigate and to compare possibly different granule structure developments, settling properties, and growth of protozoa. SBR1 and SBR2 had a working volume of 12 liters and were operated with three 8-h cycles per day. Each cycle consisted of 6 h 45 min of aeration, 5 min of settling, 5 min of effluent withdrawal, 1 h of feeding, and 5 min of resting time. Six liters of supernatant was removed, and 6-liter volumes of malthouse and brewery wastewater were fed into SBR1 and SBR2 during each cycle. The pH of the raw wastewater was adjusted to pH 8. The reactors were aerated through air bubble diffusers at a volumetric flow rate of 10 liters/min. The average organic loading rates (COD_{total}) were 2.2 kg m^{-3} day^{-1} for malthouse and 3.6 kg m^{-3} day^{-1} for brewery wastewater. Granular sludge samples (100-ml volumes) were collected from SBR1 and SBR2 and subjected to fixation procedures and microscopic investigations. Samples were taken two to three times per week 45 min prior to the end of the aeration phase. Sampling was carried out from reactor start-up to 2 weeks after granulation. Subsequently, the experiment was terminated. The working volume of SBR3 was 8 liters, with an average COD_{total} of 2.4 kg m^{-3} day^{-1}. SBR3 was operated with four 6-h cycles per day. Each cycle consisted of 5 h 20 min of aeration, 2 min of settling, 7 min of effluent withdrawal, 10 min of feeding, and 25 min of resting time. Four liters of supernatant was removed and 4 liters of synthetic wastewater was fed into SBR3 during each cycle. An airflow rate of 4 liters min^{-1} was provided during the aerobic stage of SBR3. Since SBR3 was operated as a steady-state reactor over 18 months, a 100-ml volume of sludge was discharged each day to maintain solid retention times and similar amounts of granular sludge. Granular sludge samples of SBR3 were collected two times per week 45 min prior to the end of the aeration phase. The mean pH value in SBR3 was between 7.5 and 8.0.

Sample fixation, preparation, and microbiological analysis. The complete forming process from an activated sludge floc to a mature granule was investigated and documented by light microscopy and SEM, together with digital imaging. Sludge samples were collected as described above. One part of the samples was immediately analyzed with light microscopy, whereas another aliquot was preserved by fixation for later microscopic examinations. Light microscopy was performed using a stereo microscope (Stemi SV11; Carl Zeiss AG, Oberkochen, Germany) and an inverted microscope equipped with differential interference contrast (Axiovert S100; Carl Zeiss AG) to observe the granular biofilm development and to identify protozoa. Microscopic analyses to identify filamentous bacteria, including Gram and Neisser staining, were performed as described by Eikelboom and van Buijsen (15). Samples for SEM were fixed in 2.5% glutaraldehyde in a 25 mM concentration of pH 7.0 cacodylate buffer. Further SEM sample preparations and microscopic analyses were performed as described previously (23). Samples for subsequent FISH were fixed in 2% paraformaldehyde solution or Bouin's solution (15 volumes of saturated picric acid, 5 volumes of buffered 37% formaldehyde, 1 volume of glacial acetic acid; final concentration, 50%) in accordance with previous recommendations (18). For the differentiation of bacteria involved in the granule formation process, a combination of molecular methods and microscopic techniques was applied. FISH and epifluorescence microscopy were performed to detect bacteria colonizing the ciliate stalks and to explore the microbial distribution patterns within granules. However, microscopic visualization of the core zone of a compact granule after hybridization is not possible due to the thickness (up to 10 mm) of the specimen. Therefore, a FISH protocol (18) was modified. In order to guarantee probe accessibility to the inner parts of thick granules, and for better microscopic analysis, granular biofilms had to be sectioned prior to hybridization, in a manner similar to that used for the detection of bacteria within anaerobic granules (7). The challenge in our study was to create an optimized protocol for the detection and identification of not only bacteria but also protozoa and fungi. Furthermore, it should be possible to perform FISH simultaneously with all of these microorganisms. Fixed samples were stored overnight at 4°C and gently shaken for 2 h to let the fixative infiltrate the biofilm and to preserve the inner parts of the granules. The fixative was then washed out twice in 1× phosphate-buffered saline solution while shaking on ice for 10 min. The washing step was followed by dehydration in 50%, 80%, and 100% ethanol (10 min for each step). Single granules were carefully lifted with a pipette tip, and surplus liquid was very gently removed with a paper tissue. Granules were transferred into a 2-ml cap filled with cryomedium (NEG-50 frozen section medium; Richard Allan Scientific, Kalamazoo, MI), whereby each granule was completely immersed, avoiding the inclusion of air bubbles. This embedment was performed at room temperature for a minimum of 1 h. Afterward, samples were frozen in liquid nitrogen for several minutes and stored at −80°C. Sectioning of granules into 20- to 30-μm-thick slices was performed with a microtome-cryostat (HM 500 OM; Microm, Walldorf, Germany) at −20°C. Sections were placed on precooled (−20°C) gelatin-coated microscope slides and immediately heat fixed in a hybridization

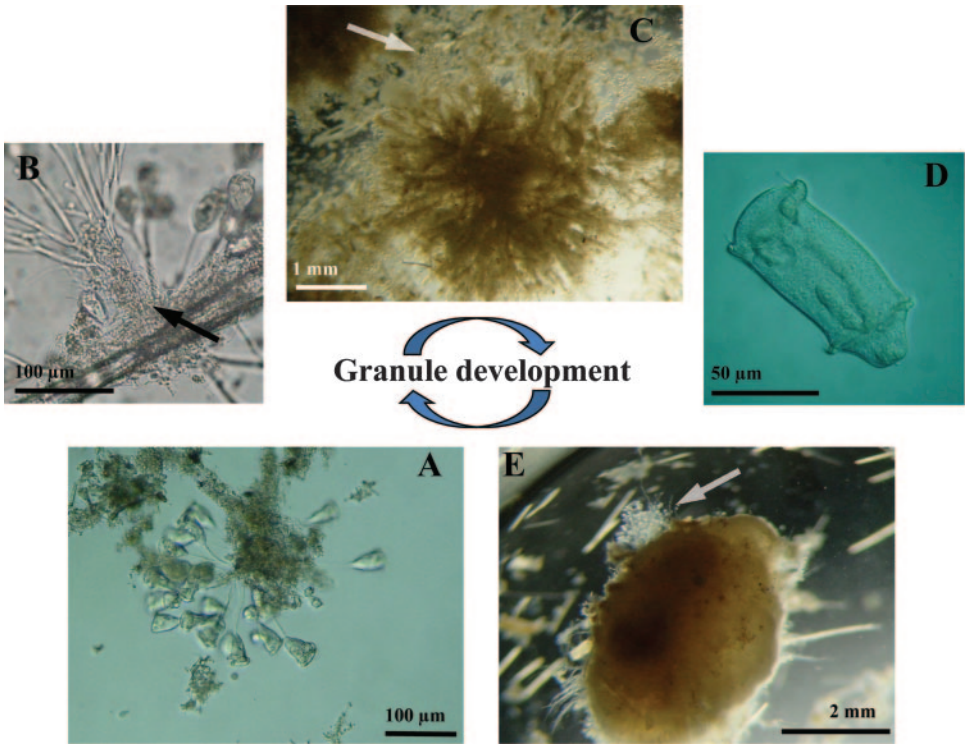

FIG. 3. Granule development supported by ciliates. Phase 1, formation of flocs. Ciliates settle on other organisms or particles (A). Bulky growth of ciliates (e.g., *Epistylis* sp.). Arrow indicates beginning colonization of ciliate stalks by bacteria (B). Phase 2, granule growth and core zone development. Zooids of the ciliates become completely overgrown by bacteria and die. Arrow marks limpid zooids of the ciliate colony. A dense core zone consisting of bacteria and the remains of ciliate stalks is formed in the center of the aggregates. Thereby, the cellular remnants of the ciliates act like a "backbone" (C). Phase 3, "mature" granule. Compact granules have formed. They can serve as a new substratum for swarming ciliate cells (D) settling on the granule surfaces (E).

oven at 46°C for at least 30 min, until the dried samples were tightly a fixed on the glass slide. To improve the adhesion of the specimen to the microscope slide during FISH, for some granules it was necessary to cover the biofilm section additionally with 0.5 to 1.0% liquid agarose (molecular grade), and subsequently with another microscope slide, in order to obtain a thin plane surface until the agarose solidified. Subsequently, to permeabilize gram-positive bacteria, samples were treated with 4×10^4 U of lysozyme at room temperature for 20 min prior to FISH. The hybridization time was extended to 2 to 3 h in order to optimize the fluorescence signals of the oligonucleotide probes bound to ribosomes within targeted cells. For the hybridization experiments, the fluorescein-labeled oligonucleotide probes EUB338, EUB338-II, and EUB338-III, which are specific for the detection of most bacteria (3, 11), the Cy3-labeled *Eukarya*-specific oligonucleotide probe EUK516 (3), and the Cy3-labeled My1574, specific for fungi (5), were applied. FISH preparations were visualized with a CLSM (LSM 510; Carl Zeiss AG) as described previously (18). Hybridizations were performed with dozens of granule sections from SBR1 and SBR3.

RESULTS AND DISCUSSION

Modified FISH protocol to determine the microbial composition of heterogeneous granular biofilms. FISH with small-subunit rRNA-directed, fluorescently labeled oligonucleotide probes is one of the most adequate and popular methods to identify bacterial species and to investigate biofilm composition and development. A modified FISH protocol as described in Materials and Methods was used to detect bacteria colonizing the ciliate stalks and to explore the microbial distribution patterns within granules. Figures 1 and 2 show oligonucleotide probe-related fluorescence signals after hybridization with the *Eukarya*-specific probe EUK516 for ciliates and fungi and with the *Bacteria*-specific probes EUB338, EUB338-II, and EUB338-III. With samples from malthouse and synthetic wastewater, all steps of the protocol worked without problems. However, proper sectioning of the bulky brewery granules from SBR2 was not possible, since they contained too many polysaccharides, which act like natural antifreeze agents. They appeared sludgy and wet and could not be sectioned with the cryotome at −20°C. However, the association of bacteria with ciliate stalks could be investigated when ciliate colonies were removed with tweezers from the surface of fixed granules and embedded in polyacrylamide prior to FISH (12).

Granule forming process. (i) Interactions of protozoa and bacteria. Granules formed within the first 2 weeks of operation. As revealed by microscopic analysis, the granule formation process can be divided into three consecutive phases (Fig. 3). The seed sludge comprised activated sludge flocs composed of bacteria, EPS, and sometimes particles (e.g., wheat glumes as in the case of SBR1, operated with malthouse wastewater). In phase 1, swarming ciliated protozoa of the subclass Peritrichia settled on sludge flocs and built new stalks (Fig. 3A). Subsequently, they started to proliferate and to form large colonies while their stalks were concurrently colonized by bacteria (Fig. 3B and 4). This colonization was additionally enhanced by the cilia beat of the ciliates, which provide a continuous nutrient flux toward the biofilm (19, 22). After a few days, several hundred ciliate cells covered the surface of each floc. Mostly tree-like colony-forming ciliates of the genera

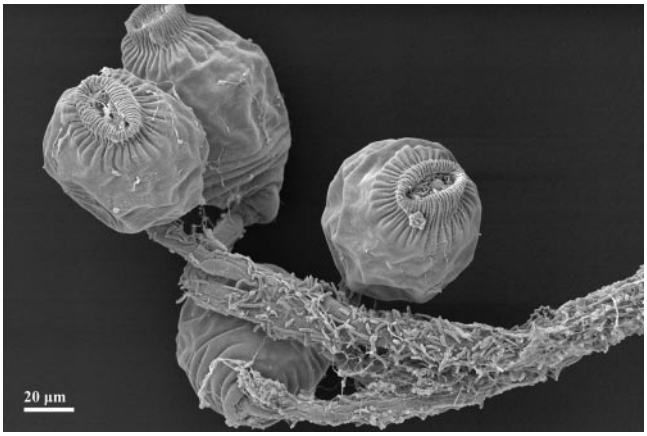

FIG. 4. Peritrichous ciliates growing in a tree-like colony. Ciliate stalks serve as backbones in the granule-forming process since bacteria use them as a substratum on which to grow. SEM.

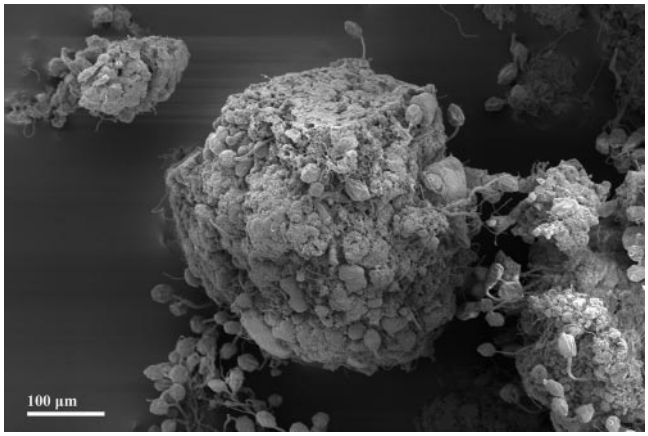

FIG. 6. Mature granule from an SBR operated with synthetic wastewater. The granule is spherical in shape and consists of bacteria and extracellular polymeric substances. It is colonized with many stalked ciliates of the subclass Peritrichia. SEM.

Opercularia and *Epistylis* occurred. During phase 2, flocs condensed and a huge growth of ciliate cells could be observed (Fig. 3C). During the formation of these bulky flocs, a core zone consisting of ciliate stalk remnants and EPS-producing bacteria occurred. The ciliate stalks served hereby as a "backbone" for granule development, since bacteria used them as a substratum to grow. The condensed aggregates were considered to be granule precursors. Subsequently, with the beginning of phase 3, the zooids (cell bodies) of the stalked ciliates were likewise colonized by bacterial cells and embedded in the expanding biofilm. After a while, they were completely overgrown (Fig. 5). Most ciliates died during this process. Some ciliate cells formed swarmers (unstalked free swimming cells) and left the biofilm to escape decay (Fig. 3D). Thus, smooth and compact bacterial granules were formed (Fig. 3E and Fig. 6). However, these mature granules were colonized step by step by the surviving swarming ciliate cells (Fig. 3E), which again formed new stalks and colonies used as a substratum for bacterial growth. Granule size may reach a steady-state size due to abrasion, washout, and floating of granules (7). This could be confirmed for granules of the SBR1 and SBR2 approach. The observations of all SBR approaches documented that peritrichous ciliates were crucially implemented in the

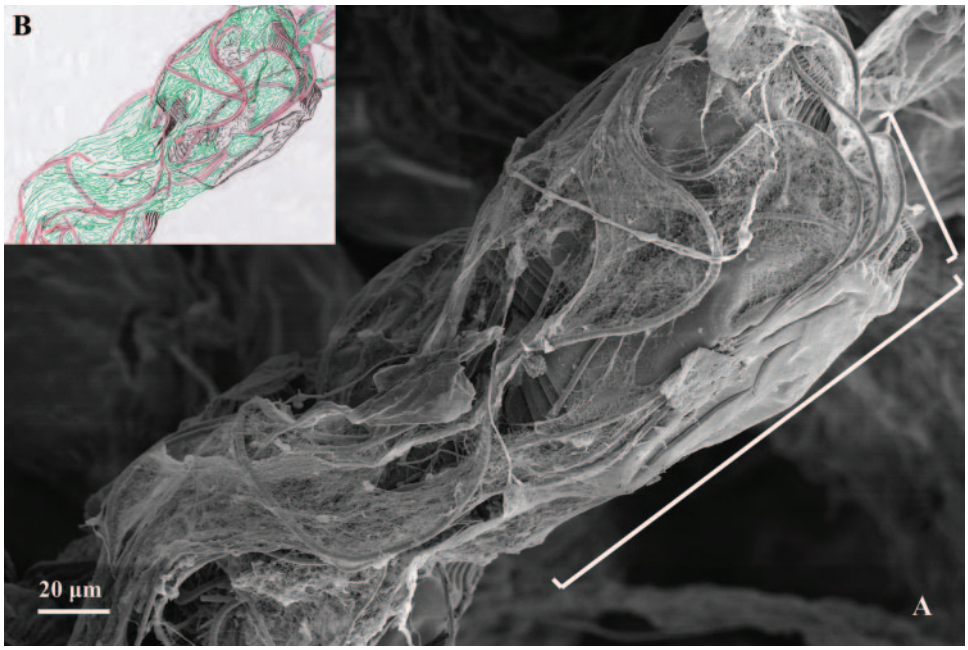

FIG. 5. Already-dead ciliates from a SBR operated with wastewater released from a brewery. Zooids (cell bodies) and stalks of two adjacent ciliate cells are completely overgrown by bacterial filaments. SEM. Brackets mark the approximate zooid boundaries of the ciliate cell on the right side (A). The scheme (B) redrawn from panel A emphasizes the boundaries between the microorganisms as ciliate cells are colored in black, covered by bacterial filaments (red) and EPS (green).

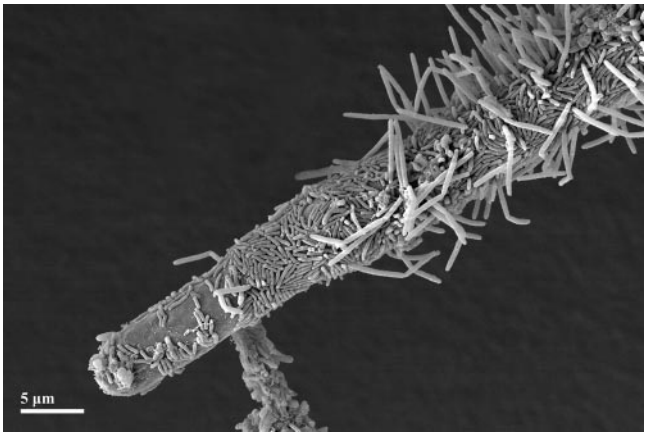

FIG. 7. Fungal filaments (hyphae) serving as the basis for bacterial colonization and therefore acting as a skeletal element in the granular structure. The active growing tip region is colonized only sparsely by bacterial cells. SEM.

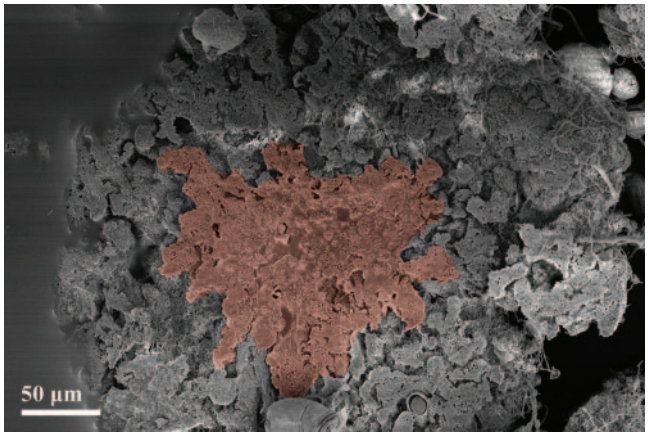

FIG. 8. Cross-section of a mature granule from malthouse wastewater. The dense core zone containing bacteria and possibly dead cell debris is recolored in red. The loose-structured fringe zone (gray) containing bacteria and ciliates encases the core zone. SEM.

granule structure-forming process, since they served as the basis for bacterial biofilm growth. Thus, the question of which specific interactions occur between ciliates and bacteria in granular biofilms arises. It is known that protozoa can excrete growth-stimulating compounds which enhance bacterial activity (34). Furthermore, it was recently reported that eukaryotes and bacteria can systematically interact with each other via small molecules (14). It remains to be investigated if such communication skills arise in the wastewater biofilm community as well and if they can enhance or stimulate granule development.

(ii) Role of fungi. A process similar to the previous one could be observed when fungi were present in the sludge samples. Compared to the ciliate-supported process, granule formation, including fungal filaments as backbones, did not result in lysis of fungi in phase 2 and a recolonization of granule surfaces in phase 3. Instead, spores of fungi germinated once in activated sludge flocs and then grew fast and continuously. Bacteria used the fungal filaments (hyphae) as well as ciliate stalks as a substratum to grow on, but fungi were never completely overgrown. The filament tips were only slightly covered with bacteria. According to a series of SEM images, the extension of this sparsely colonized, actively growing region was 5 to 30 μm long (Fig. 7). Nevertheless, dense core zones and, later, compact granules with several protruding fungal filaments developed. Microscopic analyses of sectioned mature granules revealed that their whole inner part contained remnants of hyphae. Granule formation with the help of fungi appeared first of all in the SBR3 approach with synthetic wastewater. In the SBR2, fed with brewery wastewater, no fungi except yeast cells could be found. In the SBR1, fed with malthouse wastewater, fungal spores occurred as revealed by microscopic observations. Most of them probably do not originate from seed sludge but rather from barley dust. The major portion of these spores did not germinate or build filaments to support granule formation. Only a few fungal filaments grew for some days, but they vanished thereafter. An explanation may be that many ciliates were found in the samples, possibly competing with fungi for nutrients. In the SBR3 approach, only in some cases were ciliates and fungal filaments observed growing together in the same granule. Granules normally comprised a community of either fungi and bacteria or ciliates and bacteria. Previous studies confirmed the possible structure-enhancing role of fungi in the aerobic granule-formation process (8, 17). In these studies, filamentous fungal pellets dominated the sludge population in the first 10 to 15 days and were assumed to initiate the granular biofilm structure, serving as an immobilizing matrix for bacteria. Fungal pellets fell apart later due to cell lysis in the inner part of the pellets. In contrast to these observations, fungi were detected in our study even in mature granules. They supported the development and maintenance of mature biofilms. It is also known that some fungi improve bioconversion of activated sludge (27) and perform nitrification and denitrification at high rates (21). Future studies on the diversity and function of fungi in aerobic granules could therefore yield interesting insights into sludge degradation pathways and possible biotechnological applications.

Structure of mature granules. Granules may comprise several microbial layers. This has also been reported in other case studies on anaerobic and aerobic granules (2, 13, 29, 37). Different layers were sometimes composed of distinct bacterial species with various functional tasks, such as nitrification, denitrification, or ammonia oxidation (1, 4, 38). In this study on aerobic granules, different structured zones could be identified as well. SEM analysis documented that fully grown granules always comprised a core and a fringe zone (Fig. 8). The expansions of core and fringe zones differed from granule to granule, depending on the developmental phase and wastewater type. The fringe zone could be identified as a loosely structured layer consisting mainly of bacteria and stalked ciliates or fungal filaments. In the very compact and mature granule, the fringe zone contained only ciliates or fungi growing on granule surfaces. The core zone comprised a dense mixture of bacteria and EPS. However, the rather large amount of EPS allowed bacteria to occur not only in clusters but even as isolate cells within the EPS matrix. Remnants of fungal filaments and ciliate stalks were included in the outer parts of the core zone. Depending on the size of the granules, the inner part of the

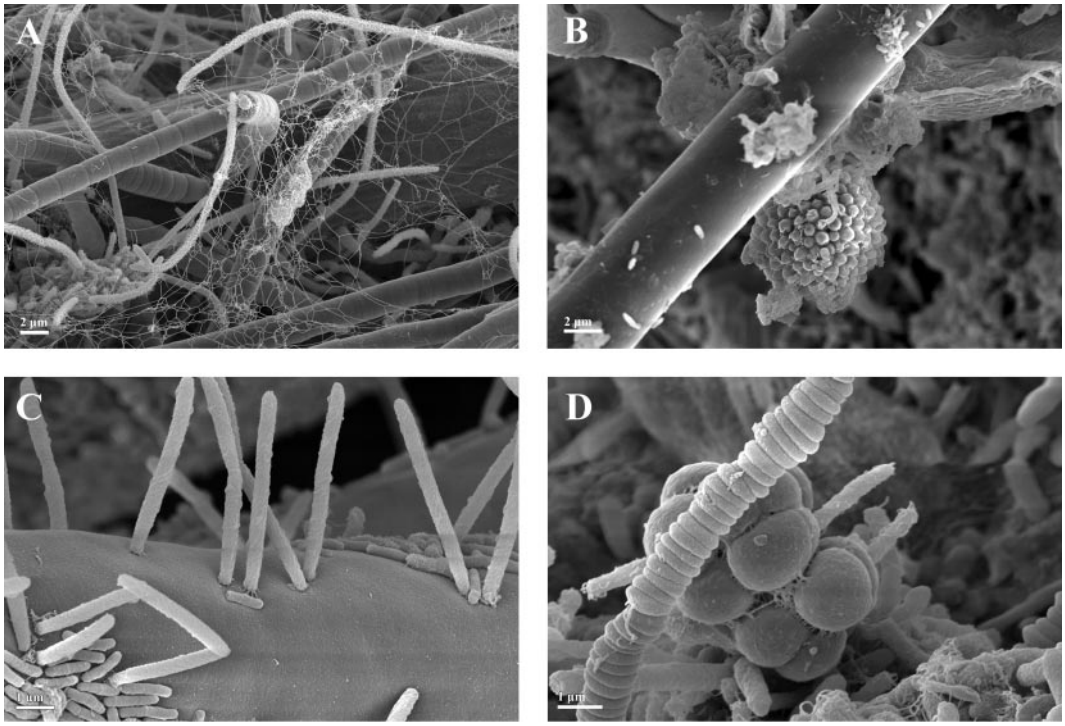

FIG. 9. Various bacterial morphotypes observed with the three reactors. Different filamentous forms (A), coccoid colony on a ciliate stalk (B), different rod shaped bacteria on a fungal hypha (C), and tetrad-arranged bacteria (D). SEM.

core zone may contain only dead cell debris, as was reported in another study (29).

Granule formation in brewery wastewater. The above-mentioned granule-forming process could be observed with all three SBR approaches, but the settling properties of granules in the SBR2 with brewery wastewater differed from those in SBR1 and SBR3. Granules of SBR2 resulted in extremely big and fluffy granules which therefore did not settle properly and caused problems like bulking in the reactor. The SBR2 was started twice, and in both cases, granules quickly developed to the phase 1 and phase 2 stages. Granule precursors of phase 2 contained many ciliate colonies. High substrate concentrations gave rise to large granules, with a diameter of 5 to 6 mm on average. The phenomenon of granule enlargement was also reported for anaerobic granules (20). However, the forming of phase 3 biofilms did not result in fast settling and compact granules, due to very intense growth of bacterial filaments. These filaments dominated the microbial composition of granules and therefore caused bulking. The dominant growth of filamentous bacteria may be considered a stress reaction of occasional oxygen deficiency due to the sometimes very high temperatures during the summer (35°C instead of the usual 20°C). Similar observations were reported previously (31). The appearance of filamentous organisms in large amounts can depend also on the substrate composition or the organic loading rate. Energy-rich substrates are known to support the proliferation of filamentous bacteria in activated sludge (32). The COD_{total} loading rate in SBR2 was higher (3.6 kg m^{-3} day^{-1}) than in SBR 1 (2.2 kg m^{-3} day^{-1}) and SBR3 (2.4 kg m^{-3} day^{-1}).

Bacterial composition of granules. Bacteria were found to be the main microbial components of the investigated granular biofilms. They occurred in a large morphological variety. Rods and cocci as well as spirilli and tetrad-arranged organisms could be found in the same granule (Fig. 9). They were embedded in a thick EPS matrix as single cells or dense clusters. Cocci often occurred in somewhat spherical clusters of several dozen cells (Fig. 9B). Colonization of fungal filaments and ciliate stalks was observed mainly for rod-shaped bacteria (Fig. 7 and 9C). Furthermore, large quantities of bacteria could be detected with SEM and FISH analysis in the digestion vacuoles of ciliates. The composition of the bacterial community and the distribution of distinct morphotypes differed depending on the wastewater type and operational set-up of the SBRs. In SBR2, mainly filamentous bacteria occurred (Fig. 9A). Gram and Neisser stains and FISH analyses showed that most of the filaments belonged to the genus *Thiothrix* or to *Sphaerotilus natans*. Synthetic wastewater granules were composed mainly of cocci, tetrad-arranged organisms (Fig. 9D), and a huge amount of EPS. EPS are generally important for granule stability. On the one hand, they cause hydration of the granule surfaces, and on the other hand, they enhance shear force stability of activated sludge flocs and granules (36). We assume that EPS are produced not only by bacteria but also by other organisms present in the observed biofilm community—in this case, especially by ciliates and fungi.

Conclusions. In the present study, the structural and main microbial composition of aerobic activated sludge granules originating from three differently operated SBRs was described. The combination of SEM, light microscopy, and

CLSM, together with a modified FISH protocol, provides a powerful tool to explore the structure of microbial granules and may be used for other biofilm formations, too. With this newly established protocol, FISH, applied to microsections of microbial granules, can now be used to monitor the development of such granules, because bacteria, ciliates, and fungi can be detected simultaneously. This is a great advantage, since eukaryotic organisms play a crucial role in the formation of granules. Stalked ciliates of the subclass Peritrichia were always involved in the process of granule development, whereas fungi were found only in some cases. It was shown that the development from the sludge floc to the mature granule takes place in three phases. The process starts with a sludge floc which proliferates to a granule precursor and results in the formation of a compact, mature granule. Ciliates and fungi serve as the main substratum in the formation of bacterial biofilms and, therefore, act as a backbone for the granules. The compactness, size, and microbial composition of granules depended on the wastewater type and the operational setup of the SBR. Many different bacterial morphotypes and large quantities of EPS could be found in the examined biofilms. The results of our study indicate that other organisms, such as ciliates and fungi, may also be involved in the formation of EPS. This finding opens a new field in biofilm research, because current studies on EPS (29) concentrate on the formation of EPS by bacteria.

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Microbial Composition and Structure of Aerobic Granular Sewage Biofilms

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Appendix B

The diversity of fungi in aerobic sewage granules assessed by 18S rRNA gene and ITS sequence analyses

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RESEARCH ARTICLE

The diversity of fungi in aerobic sewage granules assessed by 18S rRNA gene and ITS sequence analyses

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Abstract

Aerobic sewage granules are dense, spherical biofilms, regarded as a useful and promising tool in wastewater treatment processes. Recent studies revealed that fungi can be implemented in biofilm formation. This study attempts to uncover the fungal diversity in aerobic granules by sequence analysis of the 18S and 5.8S rRNA genes and the internal transcribed spacer regions. For this purpose, appropriate PCR and sequencing primer sets were selected and an improved DNA isolation protocol was used. The sequences of 41 isolates were assigned to the taxonomic groups *Pleosporaceae*, *Xylariales*, *Theleobolaceae*, *Claviceps*, *Aureobasidium*, *Candida boleticola*, and *Tremellomycetes* within the fungi. It turned out that the fungal community composition in granules depended on the wastewater type and the phase of granule development.

Introduction

Aerobic sewage granules are spherical, compact aggregates of microorganisms, mainly bacteria, but also protozoa and fungi, and extracellular polymeric substances (Morgenroth *et al.*, 1997; Beun *et al.*, 1999; Weber *et al.*, 2007). Because of their compact and dense structure, granular biofilms allow fast settling of the sludge, while the biomass is almost completely retained in the system and comprises a high and stable metabolism. Recent studies showed that an implementation of fungi in suspended granular biofilms usually occurs in sequencing batch reactors (SBRs) (Beun *et al.*, 1999; Etterer & Wilderer, 2001; Wang *et al.*, 2004; Weber *et al.*, 2007). Thereby, fungal hyphae act like skeletal elements in the structural formation process of the granules, providing a substratum for bacterial growth (Fig. 1).

Besides the use of particular fungi for industrial wastewater treatment, the overall fungal diversity in activated sludge remained mainly unidentified because these organisms were generally unwanted in conventional activated sludge processes. The filamentous hyphae were known to cause bulking problems and clog tubes and filters in the plants (Subramanian, 1983; Mudrack & Kunst, 1988). However, recent studies reported on the useful implementation of fungi in domestic wastewater treatment plants (Fakhru'l-Razi *et al.*, 2002; Guest & Smith, 2002; Molla *et al.*, 2002). Fungi contributed to sludge dewaterability and toxic resistance, COD removal, nitrification, and denitrification (Guest & Smith, 2002; Alam *et al.*, 2003; Mannan *et al.*, 2005). Some of them show even higher denitrification rates than bacteria (Guest & Smith, 2002). These observations endorse the need to investigate the fungal diversity in wastewater more closely and to integrate these organisms systematically in the treatment process to tap their possible bioconversion potential without bulking problems. For the latter task, novel treatment techniques as the above-mentioned aerobic granular sludge in SBR systems can be considered as appropriate (Weber *et al.*, 2007).

Because there is limited knowledge of the identity of fungi in granular wastewater biofilms, the aim of the present study was to examine the fungal diversity in two SBRs, operated with malthouse and artificial wastewater. Experience from numerous studies on fungal diversity in natural habitats has shown that the identification of fungi is often very complex

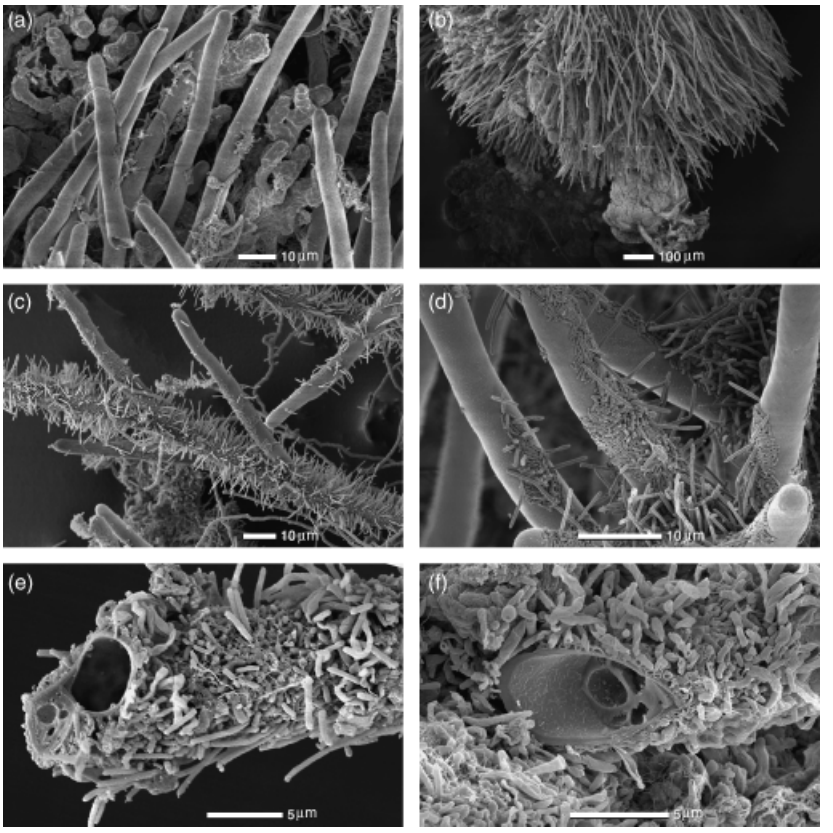

Fig. 1. Scanning electron micrographs of biofilm samples from SBR1 (malthouse wastewater; a, b) and SBR2 (artificial wastewater; c, d, e, f). Fungal filaments (hyphae) are implemented in the granule structure (a), with hyphae tips protruding from the granule surface (b). Hyphae are densely covered with rod-shaped bacterial cells both on the surface (c, d) and within the granule (e, f; visualized after cryo-fracture treatment).

due to their species richness and the high micromorphological similarities. Furthermore, these organisms exhibit multiple life-cycle types and often cannot be cultured using standard culturing techniques (Kowalchuk, 1999; Borneman & Hartin, 2000; van Elsas *et al.*, 2000). Therefore, this study applies 18S rRNA and 5.8S rRNA gene sequences and internal transcribed spacer (ITS) sequence analyses as recommended molecular methods for diversity studies on fungi within a broad taxonomic range (Kowalchuk, 1999). Because it became apparent that fungi play a meaningful role in wastewater treatment, appropriate molecular methods for the identification of these organisms will be of interest for a broad research community. Thus, this study includes improvements in methods such as DNA extraction for filamentous fungi from wastewater, evaluation and preparation of appropriate primer sets to assess the diversity of fungi in the samples and an evaluation of the phylogenetic markers used.

Materials and methods

Reactor setup and sampling

Biomass was enriched in two cylindric lab-scale SBRs. Seed sludge was obtained from the municipal wastewater treatment plant Garching, Germany. The SBRs were operated with different wastewater. SBR1 with a volume of 12 L was fed with malthouse wastewater prepared by mixing barley dust together with tap water as described elsewhere (Wilderer & McSwain, 2004). SBR2 with a volume of 9 L was operated with synthetic wastewater according to previous recommendations (Moy *et al.*, 2002). The mean pH values in the SBRs were between 7.5 and 8.0. All further physical and chemical reactor operation parameters were set as described previously (Weber *et al.*, 2007). Samples of 10 mL sludge from SBR1 were taken continuously two times per week from reactor start (early phase, floccular seed sludge) to 2 weeks after granulation (late phase, floccules have condensed to compact spherical granules with a dense core zone and a loosely structured fringe zone as described in Weber *et al.*, 2007). At the time of sampling, SBR2 was already operated since 18 months as a fully granulated 'steady state' reactor as described elsewhere (Weber *et al.*, 2007). Therefore, exclusively granular sludge in samples of 10 mL volume from SBR2 was taken twice per month over a period of 4 months. Fifty samples from SBR1 and 20 samples from SBR2 received from all granulation phases were subjected to microscopic investigations and DNA isolation. The presence of fungal spores and hyphae was monitored microscopically using a stereo microscope

(Stemi SV11, Carl Zeiss AG, Oberkochen, Germany; magnification up to $\times 105$). Single granules, which contained many fungi, were selected for further examination of their 18S rRNA gene sequence. Scanning electron microscopy was performed as described previously (Weber *et al.*, 2007).

DNA extraction

Fungi possess a diverse and very stable chemical and physical cell wall. For this reason, no universal DNA extraction method for fungi exists (Selitrennikoff, 2001; Chen *et al.*, 2002; Yeo & Wong, 2002; Karakousis *et al.*, 2005). DNA extraction was carried out applying three different methods in order to compare the received DNA yield and to identify the most adequate isolation protocol for fungi from granular sludge samples, using: (1) The Dneasy® Tissue Kit (Qiagen GmbH, Hilden, Germany), following the guidelines of the manufacturer for 'purification of genomic DNA from yeast', which includes enzymatic lysis of the fungal cell wall with Lyticase. (2) A bead beater to break cell walls mechanically following the protocol 'Simultaneous RNA/DNA extraction from soil samples and pure cultures' (Lüders *et al.*, 2004). (3) A modified protocol, which combines enzymatic and mechanical cell wall lysis, implementing a bead-beating step in the kit protocol after enzymatic treatment of the cells with Lyticase. The success of genomic DNA purification was proved by documentation with 1% agarose gel electrophoresis. Seventy samples were subjected to DNA isolation.

PCR and cloning

Fungal DNA was specifically amplified from the crude DNA extract with fungi-specific primers targeting the 18S rRNA gene and the conterminal ITS region, including ITS1, ITS2, and the 5.8S rRNA gene. The organization of the fungal rRNA operon is shown elsewhere (White *et al.*, 1990). The target sites of the primers were chosen to receive preferably the complete 18S rRNA gene sequence and the ITS region. Primers and target sequences were selected and evaluated using the ARB software (Ludwig *et al.*, 2004; http://www.arb-home.de) by comparing $> 41\,000$ rRNA gene sequences of prokaryotes and eukaryotes. Different primer pairs have been selected for the best possible phylogenetic coverage of the fungal diversity in the samples. The forward primers EF4 (Smit *et al.*, 1999) and NS1f (White *et al.*, 1990), both targeting the 18S rRNA gene, and the reverse primers ITS4r (White *et al.*, 1990) and LR1r (Vilgalys & Hester, 1990), both targeting the 28S rRNA gene, were selected for PCR amplification. Additionally, the forward primer EF60f (5′-TGTCTAAGTATAAGCAATT-3′, the homologous primer position for reference organism *Saccharomyces cerevisiae*, EMBL accession number V01335, for the target molecule 18S rRNA gene is 60 bp starting from the 5′-end) was designed and used for this study. EF60f was constructed for this study as a new fungi-specific forward primer. The primers FF390r (Vainio & Hantula, 2000), 18S-1101-CIL-S-f (Fried, 2002), and the primers M13f and M13r (specific for the TOPO TA cloning vector, TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) were selected for sequencing purposes. Considering the ARB probe match tool, the primers EF60f and EF4f were fungi-specific while NS1f may also detect some protozoan species. The amplification of the 18S rRNA gene, together with the conterminal ITS region, was carried out with NS1f/ITS4r, NS1f/LR1r, EF4f/ITS4r, and EF60f/ITS4r to select for the best primer combinations. PCR was carried out with genomic DNA samples using the TaKaRa ExTaq™ polymerase system (TaKaRa Shuzo Co., Otsu, Japan) according to Chou *et al.* (1992). The PCR reaction of every sample was performed with 5 μL ExTaq™ buffer ($10\times$), 4 μL dNTP-Mix (2.5 mM per nucleotide), 0.5 μL ExTaq™ polymerase enzyme, 0.5 μL primers, each 50 pmol μL^{-1}, 100 ng of DNA, and water added to a total volume of 50 μL. PCR included an initial denaturation step (94 °C, 10 min), followed by 30 cycles (94 °C, 1-min denaturation; '*x*' °C, 1-min primer annealing; 72 °C, 3-min elongation), and a final elongation step (72 °C, 10 min). The optimal annealing temperature ('*x*') for every primer pair was determined by performing gradient PCRs in steps of 0.5 and ± 4 °C around the primer-specific melting temperature (T_m). The optimal annealing temperatures determined for the primer pairs are 53 °C for NS1f/ITS4r, 60 °C for EF4f/ITS4r, and 55 °C for EF60f/ITS4r. Subsequent to PCR, a clone library of PCR fragments with 145 clones was generated for sequencing purposes, using the TOPO TA cloning kit. From 145 PCR fragments, 119 were successfully cloned.

Sequence analysis

Sequencing of 119 clones was performed using the SequiTherm EXCELRII DNA Sequencing Kit-LC (Epicentre, Madison, WI) and using an LI-COR Global IR2 DNA sequencer (66 cm; LI-COR Biosciences, Bad Homburg, Germany). The primer combinations 18S-1101-Cil-S-f/M13r and M13f/FF390r were used to receive the complete sequence of the 18S rRNA gene and the ITS region. FF390r is a fungi-specific primer. 18S-1101-Cil-f is a forward primer that was already successfully used for sequencing the 18S rRNA gene of eukaryotes, for example ciliated protozoa (Fried, 2002). After verification of this primer in the database, it became apparent that it can also be used for sequencing of fungal 18S rRNA gene sequences. Incomplete sequences and, after a first phylogenetic comparison of all received clone sequences, those of protozoan origin were discarded. The sequences from 41 of 65 fungal clones, which were originally received with the PCR primer pairs EF4f/ITS4r and EF60f/ITS4r, were used for further phylogenetic

analysis. Raw sequence data were analyzed with the E-SEQ software (LI-COR Biosciences). All sequences were tested for chimeras with an ARB intrinsic software for the chimera check of prokaryotes and eukaryotes. No chimeras were found.

Phylogenetic analysis

Phylogenetic analysis was performed with 41 sequences using the alignment and tree calculation methods of the ARB software package. The new 18S rRNA gene sequences were added to an existing ARB-alignment for the 18S rRNA gene sequence with the ARB primary and secondary structure editor. For the ITS region sequences, an extra ITS database was created, together with public sequences obtained from the EMBL database (http://www.ebi.ac.uk/embl). A new alignment was created with the help of CLUSTAL W implemented in ARB. The ITS region and the 18S rRNA gene sequence were separately aligned. Likewise, phylogenetic trees of the 18S rRNA gene sequence and ITS region sequences of all 41 clones were calculated separately and compared afterwards. Phylogenetic consensus tree constructions were based on distance matrix, maximum parsimony, maximum likelihood, and tree puzzle methods following the recommendations for phylogenetic analysis as described previously (Ludwig *et al.*, 1998). A minimum similarity filter was chosen, which only retained positions conserved in at least 50% of the selected sequences.

Results and discussion

Phylogenetic analysis of the clones

Because very little is known about the diversity of fungi occurring in SBR systems, we attempted to characterize the fungi of aerobic sewage granules from two different wastewater treatment approaches. Both difficulties in culturing and in micromorphological differentiation required the application of molecular methods to assess the fungal diversity (Kowalchuk, 1999). The use of 18S rRNA gene analysis in the field of phylogeny is common and well established for many eukaryotic organisms (White *et al.*, 1990; Aleshin *et al.*, 1998; Smit *et al.*, 1999; Borneman & Hartin, 2000; Fried, 2002; Hughes & Piontkivska, 2003; Schaap *et al.*, 2006) and was applied for this study. Forty-one rRNA gene clones were received from malthouse and artificial wastewater samples. Their overall phylogenetic classification is shown in Fig. 2. Within both SBR wastewater approaches, representatives of the *Ascomycota* and *Basidiomycota* were identified. Sequences representing the *Basidiomycota* were assigned exclusively to the *Tremellomycetes*. Sequences representing the *Ascomycota* were assigned to the taxonomic groups *Pleosporaceae*, *Xylariales*, *Theleobolaceae*, *Claviceps* and relatives, *Aureobasidium* and relatives, and to the *Candida boleticola* cluster. Artificial wastewater samples contained representatives of *Tremellomycetes*, and *Aureobasidium* and relatives. The fungal diversity in malthouse samples was clearly higher. In these samples, all of the above-listed taxonomic groups of the *Ascomycota* and *Basidiomycota* were identified. Some studies reported that the fungal 18S rRNA gene sequence can only be used for analysis of species that are distantly related (White *et al.*, 1990). Here, the use of 18S rRNA gene sequence analysis allowed a phylogenetic differentiation down to the levels of families or genera. Some clones representing organisms assigned to the *Pleosporaceae* clustered closely with *Phoma herbarum* and *Pleospora rudis* and some others indicated a monophyletic group related to *Leptosphaeria* sp. Two clones from artificial wastewater and two clones from malthouse wastewater assigned to the *Aureobasidium* and relatives cluster showed sequence similarities to rRNA primary structures from *Aureobasidium pullulans* and *Discosphaerina phagi*. A member of the *Theleobolaceae* indicated by clone 5 clustered with *Theleobolus stercoreus* and a relative of *Monographella nivalis* within the *Xylariales* was represented by clone 15. A very clear relationship with all species of the genus *Candida* currently listed in the GenBank database was found for a

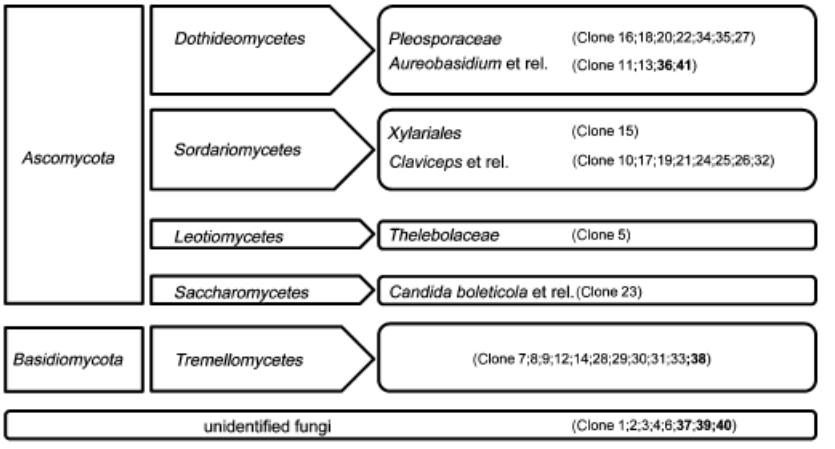

Fig. 2. Overview of the affiliation of clone sequences obtained from malthouse (marked in bold) and artificial wastewater samples to different fungal taxonomic groups according to 18S rRNA gene sequence analysis. Most clones are assigned to different groups of the *Ascomycota*. Clone sequences representing the *Basidiomycota* were assigned exclusively to the *Tremellomycetes*. Some clones derived from artificial wastewater could not be assigned to any defined group of fungi and are thus, listed as unidentified fungi.

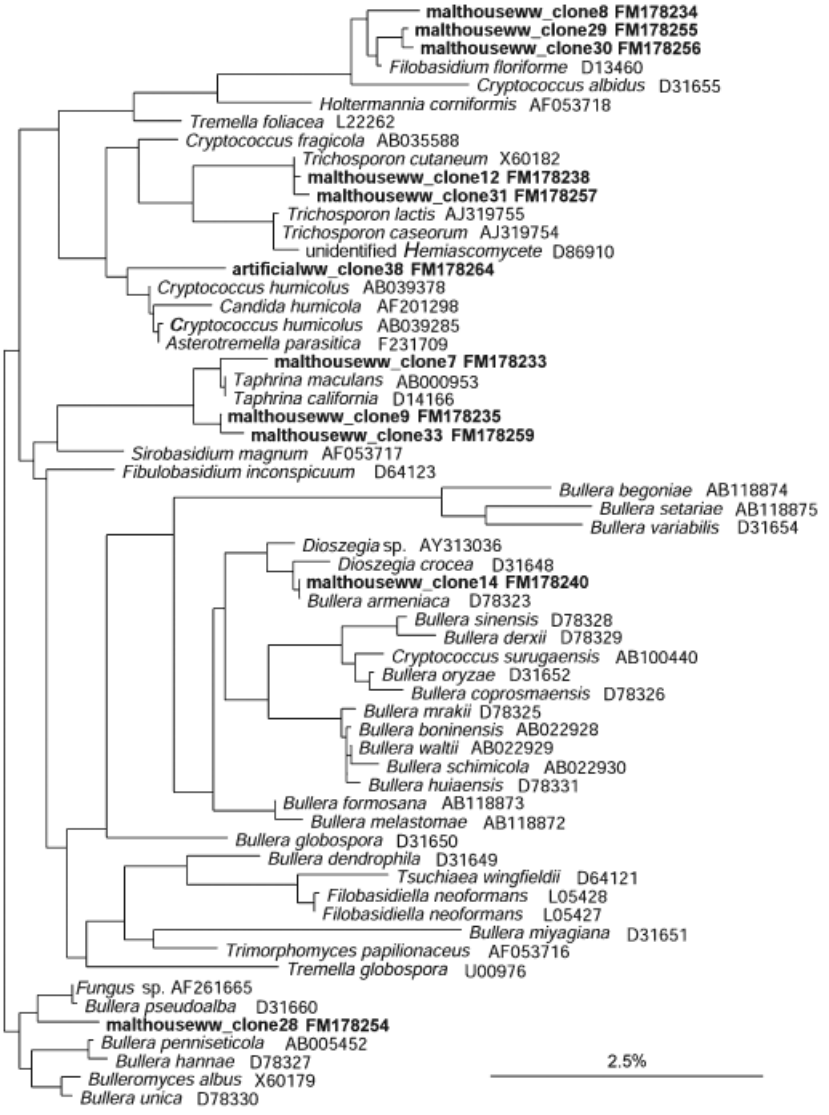

Fig. 3. Maximum likelihood-based tree reflecting the phylogeny of the *Tremellomycetes* based on 18S rRNA gene sequence comparisons. Bold indicates the sequences obtained during this study. Numbers behind taxonomic names represent EMBL sequence accession numbers. The scale bar indicates 2.5% estimated sequence divergence. Note: two representatives of the genus *Taphrina* (*Ascomycota*, *Taphrinomycotina*) group within the *Tremellomycetes*, which is not in accordance with its systematic assignments. Therefore, the GenBank sequence entries of *Taphrina maculans* AB000953 and of *Taphrina californa* D14166 should be revised.

yeast taxon represented by clone 23. However, no stable intragroup branching pattern could be found. Therefore, the respective organism could not be clearly assigned to its next phylogenetic neighbors within the *C. boleticola* cluster. An example of a phylogenetic tree representing the 18S rRNA gene-based assignment of clones within the *Tremellomycetes* is shown in Fig. 3. Some of these clone sequences clustered with homologous sequences from *Filobasidium floriforme*, *Trichosporon cutaneum*, *Cryptococcus* sp., *Bullera* sp., and *Dioszegia* sp. Within this cluster, two representatives of the genus *Taphrina* (*Ascomycota* and *Taphrinomycotina*) can also be found. Systematically, *Taphrina* is assigned to the *Ascomycota*, *Taphrinomycotina* and does not fit into the *Tremellomycetes*. Thus, the GenBank sequence entries of *Taphrina maculans* AB000953 and of *Taphrina californa* D14166 must be revised.

In both approaches, some fungal clone sequences, which could not be clearly assigned to those from any defined fungal taxon, were found. The overall sequence similarities of this unidentified group to their next phylogenetic neighbors *Rozella allomyctis*, *Physioderma dolicii*, and *Physioderma maculare* are 87.6%, 88.8%, and 87.2%, respectively.

In addition to 18S rRNA gene analysis, ITS markers were used for a more refined differentiation because the ITS regions by themselves were reported as highly variable and recommended to be adequate for analyses with high phylogenetic resolution (Chen *et al.*, 2001; Manter & Vivanco, 2007). In our study, the phylogenetic assignment of the 18S rRNA gene sequences to taxonomic groups was always consistent with the assignment of the associated ITS sequences in these taxa. However, the intragroup resolution power as well as branching pattern significance and stability

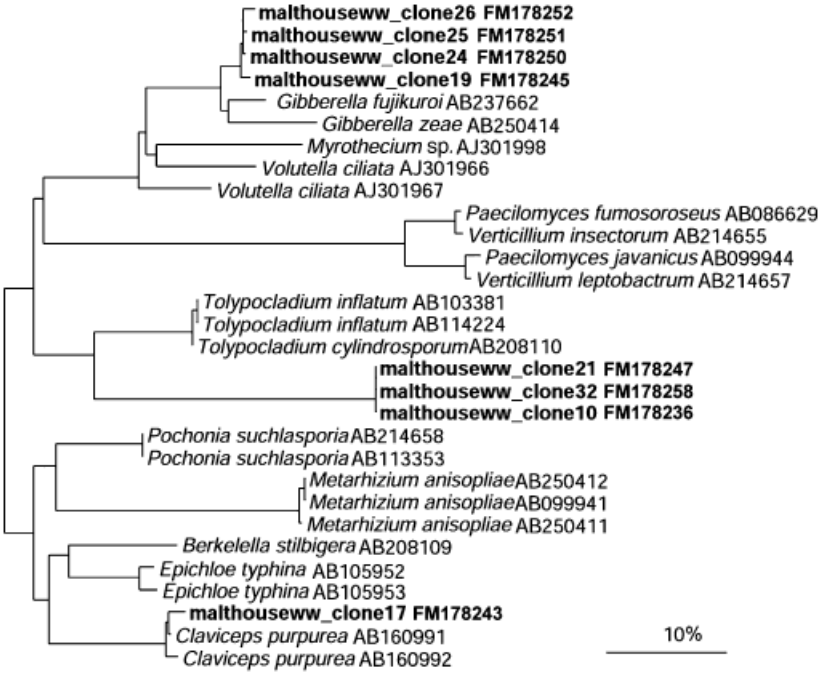

Fig. 4. Maximum likelihood-based phylogeny of the *Claviceps* et rel. cluster based on ITS sequences. Malthouse wastewater clones formed a monophyletic group with *Tolypocladium* sp. or with *Claviceps purpurea*. Some clones clustered with *Gibberella* spp. Bold indicates sequences obtained during this study. Numbers behind taxonomic names represent EMBL sequence accession numbers. The scale bar indicates 10% estimated sequence divergence.

are different for the two markers and a generally higher discriminatory power is expected for the ITS sequences. This could be shown for the *Claviceps* and relatives group (Fig. 4). The ITS tree of *Claviceps* and relatives contains one clone sequence with similarity to *Claviceps purpurea* and three clone sequences, clones 21, 32, and 10, representing organisms of a monophyletic group with *Tolypocladium* spp. The sister group status of *Tolypocladium* and the three clone sequences was significantly supported in the ITS tree, but not resolved in the 18S rRNA gene sequence phylogeny (data not shown). However, for all other ITS clone sequences, no refinement compared with 18S rRNA gene trees was possible because the number of publically available ITS reference sequences for the respective groups is still too small. Considering the current growth of available data and the enormously fast sequencing advances in molecular biology, the use of ITS sequences is also regarded as an adequate and important tool supporting and extending rRNA gene-based phylogenetic analysis. However, before it exerts its full use, there is an indispensable need to expand the dataset, especially for complex environmental samples that contain many phylogenetically quite diverse fungi. This study provides 41 new ITS region sequences and represents one more step toward the aim of an extended ITS database.

Fungi occurring in early- and late-phase granular biofilms

The use of granular sludge technology creates new possibilities for the application of fungi in wastewater treatment. Fungal hyphae cause no bulking problems when implemented in compact sewage granule biofilms. Concurrently, the manifold metabolic characteristics of fungi can be used for wastewater treatment purposes. Therefore, especially those fungi were of interest, that were present in the early phase with floccular seed sludge and also in the late phase after granular biofilm development. These fungi may contribute particularly to the biofilm structure of the sludge granules. The results of this study showed that most of the investigated fungi could be found before and after granulation.

In malthouse wastewater *Claviceps* and relatives, *Tremellomycetes* and *Pleosporaceae* were found in both phases. Some representatives of the order *Tremellomycetes*, which were the only fungi additionally identified in the granular samples from artificial wastewater, are saprotrophic organisms that find an adequate environment for growth in wastewater. Several other clones indicated organisms, assigned to the *Pleosporaceae*, which clustered, for example, with *P. herbarum*, which is a plant pathogen. They may be originally derived from the barley dust that was continuously supplied to the reactor.

Representatives of *Aureobasidium* and relatives could be found in malthouse wastewater samples before, but not after granulation. However, they were detected in granules from artificial wastewater when fungal filaments were isolated directly from single granules and identified by sequence analysis as representatives of the *Aureobasidium* and relatives taxonomic group. These clones showed high sequence similarities to *A. pullulans*, which occurs often in activated sludge (Subramanian, 1983). Here, they contributed to the

granular biofilm structure. The hyphae serve as a substratum for bacteria to grow and act like skeletal elements in the granule architecture (Weber *et al.*, 2007; Fig. 1). Hence, the interactions of fungi and bacteria represent another important aspect in biofilm development. Fungal–bacterial interactions were already investigated in the fields of medical science, food microbiology, or ecology of the soil and rhizosphere, and it was shown that bacteria can enhance or suppress the filamentation of fungi (Wargo & Hogan, 2006). Therefore, future research of complex wastewater biofilms should also focus on such bacterial–fungal relationships.

Methodological findings and improvements

This study provides some methodological aspects regarding DNA extraction and PCR primers.

We tested three different DNA extraction protocols to isolate fungal DNA from wastewater samples. The visualization of purified eukaryotic and prokaryotic genomic DNA on agarose gels showed that DNA extraction was feasible with all described methods. However, the yield of amplified 18S rRNA gene sequences and ITS fragments varied with the DNA isolation method. Fungal DNA fragments could successfully be amplified from 44% of the tested samples that were handled with the bead beater isolation protocol. After DNA purification with the DNA Tissue Kit or the modified protocol, a PCR fragment yield of 60% could be achieved, respectively. Therefore, the enzymatic and the combined enzymatic–mechanical approach can be used for the extraction of fungal DNA from wastewater samples. However, the authors recommend the use of the combined approach because enzymatic extraction alone may be sometimes poor for filamentous fungi. Thus, according to another study on the efficiency of DNA extraction methods for medically important fungi (Karakousis *et al.*, 2005), we succeeded with the use of a commercial enzymatic kit with an implemented mechanical step.

Following the successful DNA extraction, adequate PCR primer pairs were tested. The primers used for PCR should fulfill three requirements: (1) they should cover the phylogenetic spectrum in the database as far as possible, (2) discriminate against other eukaryotes, and, due to their target sites, (3) allow amplification of the complete fungal 18S rRNA gene and the conterminal ITS region. Some previously published primers (NSA3, NSI1, 58A1F, 58A2F, 58A2r, 58A1F, 58A2F, and 58A2r; Kendall & Rygiewicz, 2005) could not be used because they amplify only parts of the 18S rRNA gene and the ITS regions. Additionally, due to the highly conserved nature of the 18S rRNA gene, no previously published primer pair could be used, nor could a new one be designed that would cover all fungal 18S rRNA gene sequences in the database and exclude other eukaryotic organisms. Thus, we followed the recommendation of Kendall & Rygiewicz (2005): it is necessary to use multiple primer sets for the identification of fungi in natural habitats to include the range of organisms under study.

The four primer combinations described in Materials and methods were tested for amplification of the 18S rRNA gene and the ITS regions. NS1f is known to detect a wide variety of fungi and other eukaryotes (White *et al.*, 1990). It was tested in our database with the probe match tool of ARB for the specificity for fungi. The primer matched without any mismatch most fungal sequences and additionally some protozoa, including species of the genera *Epistylis*, *Vorticella*, and *Carchesium* (phylum *Ciliophora*), which can often be found in wastewater. Nevertheless, we attempted to amplify fungal sequences with the primer combinations NS1f/ITS4r and NS1f/LR1r taking into account that some amplified DNA would be of protozoan origin because several protozoa were observed in our samples. However, the use of NS1f/LR1r did not lead to the amplification of DNA fragments and nearly all sequences received with NS1f/ITS4r turned out to be protozoan DNA even under modified PCR conditions with different annealing temperatures. Therefore, we decided that NS1f was not useful to detect fungi in our samples.

EF60f/ITS4r and EF4f/ITS4r were adequate for the specific amplification of fungal DNA. The EF60f primer was constructed during this study taking into consideration that the best possible coverage of fungi in our database was guaranteed and no protozoan rRNA gene sequence was amplified. Additionally, EF60f should allow the amplification of all fungi in the database that could not be covered with EF4f. In our database, EF60f and EF4f together covered 1372 different fungal sequences (*c.* 70%) from a total of 1991 without any mismatch. Performing PCR with EF60f and EF4f resulted in 18S rRNA gene sequence fragments of *c.* 1740 and 1600 bp, respectively. The length of the ITS region can vary between 50 and 1050 bp (Ranjard *et al.*, 2001) and was between 156 and 721 bp for our clones.

The results showed that sequences received with the primer pairs EF60f/ITS4r and EF4f/ITS4r from the same samples were assigned to different taxa, except for representatives of *Claviceps* and relatives, which could be amplified with both forward primers. The use of EF60f combinations did not lead to the amplification of DNA fragments from artificial wastewater samples, implying that specific target organisms for that primer could not be detected in these samples. All 18S rRNA gene sequences and ITS fragments from artificial wastewater were amplified only with the primer combination EF4f/ITS4r. A previously described preference of EF4f primer combinations to target the 18S rRNA gene of *Ascomycota* extracted from soil (Kowalchuk & Smit, 2004) could not be observed for the wastewater samples in our study. In contrast, all representatives of the *Basidiomycota* were detected with EF4f primer

combinations, and representatives of the *Ascomycota* were mainly identified with EF60f ones.

Conclusions

This study represents a combined approach to assess the diversity of fungi and their possible role in granular SBR biofilm samples by improving the required molecular techniques such as DNA extraction and the application of adequate primer sets for 18S rRNA gene and ITS amplification of fungi. The sequence data were used for phylogenetic analysis.

Regarding the common knowledge of the metabolic potential of fungi and the high fungal diversity presented in this study, the role, diversity, function, and application of fungi in activated sludge processes should be further examined. It was shown that some of the identified fungi might be involved in the structural buildup of granular sewage biofilms because they could be found before and after granulation and were implemented in the granules. The specific development of technical procedures to integrate fungal filaments in compact granular sludge will support the purposeful use of fungi in wastewater treatment.

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Appendix C

Diversity, population dynamics and association of protozoa and bacteria in aerobic sludge granules from industrial wastewater sequencing batch reactors

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Experimental work: Silvia Weber
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Scientific advice for the diversity and ecology of bacteria: Hilde Lemmer
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Title: **Diversity, population dynamics, and association of protozoa and bacteria in aerobic sludge granules from industrial wastewater sequencing batch reactors**

Running title: Diversity and association of ciliates and bacteria in aerobic granules

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Abstract

Aerobic sludge granules are spherical suspended biofilms which can strongly improve purification efficiency and sludge settling in wastewater treatment processes. Recent studies revealed that stalked ciliates of the subclass *Peritrichia* are key role players in the formation and structure of aerobic sludge granules. This study enlightens the diversity of protozoa in the

granule formation process and associated bacteria, situated both inside the granules and on ciliate stalks. Comparative microscopic analyses and fluorescence in situ hybridizations (FISH) were performed with sludge from sequencing batch reactors (SBRs) operated with dairy, malting, brewery, and synthetic wastewater. Results showed high ciliate diversity in flocculent seed sludge which diminished during granule formation and ended up in the dominance of single species of the genera *Epistylis*, *Opercularia*, and *Vorticella*. Analysis of the bacterial diversity in SBR samples showed differences of bacterial genera between the different types of wastewater. Overall, mostly the taxa *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* were found. Rod-shaped bacteria belonging to the *Gammaproteo-*, *Betaproteo-*, and *Actinobacteria* were found to be associated with ciliate stalks while filamentous bacteria competed with the *Peritrichia* for settling sites on the granule surface. This study is a further step towards the understanding of microbial communities in granular sludge.

Keywords

Wastewater, aerobic granular sludge, diversity, FISH, ciliates, bacteria

1 Introduction

Wastewater-borne granular biofilms are until today not satisfactorily recordable. It is still unclear which parameters are decisive to shape and influence their structure and composition. Some studies propose that wastewater biofilms are determined by substrate gradient and shear forces others suppose that not technical parameters but the type of wastewater is decisive for biofilm composition (van Loosdrecht et al., 1997; Batstone & Keller, 2001; Bathe et al., 2005). Another study proposes that wastewater biofilms never reach a real steady state and could theoretically only be reproduced until the first sloughing or abrasion event but not afterwards (Lewandowski et al., 2004). Otherwise it is known that aerobic/anaerobic cycles

promote granule formation. However, the diverse studies point out that no universally valid concept exists until now which describes the main properties and causes of granular biofilm development in aerobic sequencing batch reactors (SBRs).

1.1 Characterization of aerobic granular sludge. Aerobic sludge granules are compact spherical biofilms providing high purification efficiency and crucially enhancing sludge settling in wastewater treatment processes (Morgenroth et al., 1997; Venugopalan et al., 2005; Zheng et al., 2006; Wang et al., 2007). Such granules are considered to be a special biofilm formation, composed of self-immobilized cells building a 'biofilm in suspension'. They consist of mainly bacteria embedded in a matrix of extracellular polymeric substances (EPS) but also protozoa and fungi (Beun et al., 1999; Wang et al., 2004; Weber et al., 2007; Lemaire et al., 2008; Weber et al., 2009). Granules comprise a high and stable metabolism, are able to withstand fluctuating organic loading rates which arise quite often in real-life remediation scenarios, and exhibit a very good resilience to toxin shocks due to their protecting EPS matrix (Wingender et al., 1999). Overall, they represent a future-oriented activated sludge technique which copes with both increasing requirements of complex industrial wastewaters and space shortages in industrialized areas.

1.2 Influence of ciliated protists. Several studies on ciliates which generally constitute a natural part of the microbial community in activated sludge systems demonstrated that these organisms fulfil a wide variety of important tasks in biomass conversion and water clarification processes. They serve as bioindicator organisms, are very efficient in particle elimination, and can excrete extracellular polysaccharides which support floc and biofilm aggregation. Stalked ciliates contribute to configure the structure of aerobic granular wastewater biofilms (Curds et al., 1968; Martin-Cereceda et al., 1996; Ratsak et al., 1994;

Roessink & Eikelboom, 1997; Nicolau et al., 2001; Weber et al., 2007; Arregui, 2007; Lemaire et al., 2008).

A recent study showed the development of granules in aerobic SBRs in presence of stalked ciliates to take place in three phases in which activated sludge flocs condense and grow to mature biofilms (Weber et al., 2007):

In phase 1 swarming peritrichous ciliates settle on activated sludge flocs, build up new stalks and form large, often treelike colonies. During phase 2, excessive growth of peritrichous ciliates is observed with their stalks being concurrently colonized by bacteria in large numbers. In the inner part of these granule precursors, ciliate stalk remnants build a backbone for a dense bacterial core zone. In phase 3, ciliates are mostly overgrown by bacteria whereby smooth and compact mature granules are formed.

1.3 Aims of this study. This study elucidates the microbial community composition of aerobic granular biofilms received from five lab-scale SBRs operated with dairy (D), malting (M), brewery (B), and synthetic (S1, S2) wastewater. The diversity and population dynamics of ciliates was observed during and after the development of sludge granules and key role players among these organisms were determined. Since association of bacteria and ciliate stalks was detected in the initial studies on SBRD, a comprehensive fluorescence in situ hybridization (FISH) survey was carried out for the following set-ups with SBRM, SBRB, SBRS1, and SBRS2 to overview the bacterial diversity during and after granule formation. Furthermore, FISH with granule sections should prove if bacteria exhibit specific layers inside the granules. Bacteria associated with the stalks of ciliates or fungal hyphae were to be identified. Moreover, possible relationships, i.e. specificity of bacterial taxa being attached to the ciliate stalks, were to be verified. Possible competition between bacteria and ciliates were to be investigated, too. Thus, this study provides a further step towards clarifying structure,

diversity, and interaction of a highly diverse biocenosis of prokaryotic and eukaryotic organisms within complex wastewater biofilms.

2 Materials and Methods

2.1 Reactor set-up and sampling frequency. Biomass was enriched in four SBRs. The SBRs were operated with different wastewater. SBRD with a volume of 12 l was fed with dairy wastewater (Weihenstephan, Freising, Germany) as previously described (Schwarzenbeck et al., 2005). SBRM with a volume of 12 l was fed with malthouse wastewater prepared by mixing barley dust with tap water as described elsewhere (Schwarzenbeck et al., 2004). SBRB with a volume of 12 l was operated with raw wastewater from a brewery (Weihenstephan, Freising, Germany) at the same set-up as SBRM. These three reactors were aerated through air bubble diffusers at a volumetric flow rate of 10 L min^{-1} and were operated until the formation of granules. SBRS1 and SBRS2 with a volume of 9 L each were operated as 'steady state' reactors with fully granulated sludge. They were fed with synthetic wastewater based on the recommendations of Moy et al. (2002) and aerated with 4 L min^{-1} and 6 L min^{-1}, respectively, to analyze if a different aeration flow influences the community composition. Flocculent seed sludge for all reactors in this study was obtained from the municipal wastewater treatment plant Garching, Germany. The mean pH value in the SBRs was between 7.5 and 8.0. The average COD_{total} loading rates were 2.8 kg m^{-3} d^{-1} for SBRD, 2.2 kg m^{-3} d^{-1} for SBRM, 3.6 kg m^{-3} d^{-1} for SBRB, and 2.4 kg m^{-3} d^{-1} for SBRS1 and SBRS2. The COD was measured spectrophotometrically (Hach-Lange, Düsseldorf, Germany). Samples of 10 mL sludge from SBRD, SBRM, and SBRB were taken twice weekly from reactor start-up (flocculent seed sludge) until two weeks after granulation. Samples of 10 mL fully granulated sludge from SBRs S1 and S2 were taken twice monthly over a period of four months. Since SBRs S1 and S2 were already fully granulated at the time of analysis, no flocculent sludge was sampled.

2.2 Granule sample preparation. The sludge samples from all SBRs were immediately subjected to light microscopic investigations. Samples for subsequent FISH experiments were fixed in 2% paraformaldehyde solution or Bouin's solution (saturated picric acid:buffered 37% formaldehyde:glacial acetic acid = 15:5:1 vol:vol; 50 % final concentration) in accordance with previous recommendations (Fried et al., 2002; Weber et al., 2007). 8-10 granules of each sample were sectioned for FISH analysis into 20-30 µm thick slices with a microtome-cryostat at -20°C (HM 500 OM; Microm International, Walldorf, Germany), placed on precooled (-20°C) gelatin-coated microscope slides and immediately heat-fixed as previously described (Weber et al., 2007). Approximately 5 granules per sample were split with tweezers to obtain pieces from the granule surface which contained many treelike ciliated protist colonies. These selected pieces were placed on gelatin-coated slides for identification of bacteria attached to protozoan stalks. 20 granules of each sample were homogenized in 2 mL-caps, each cap containing 500 µL ice-cold 1x phosphate-buffered saline solution (PBS) and a single granule. Homogenization was done twice for 5 s using an Ultra-Turrax® Homogenizer (IKA, Staufen, Germany). Subsequently, the homogenate was centrifuged and washed with PBS. The pellet was re-suspended in 500 µL PBS-EtOH solution (PBS:EtOH = 1:1 vol:vol) and stored at -20° C. Homogenized samples were easier to prepare and to handle than granule sections and were used for first FISH tests to check probe specificity.

2.3 Identification of ciliates. To observe and identify organisms light microscopy was performed using a stereo microscope (Stemi SV11, Carl Zeiss, Oberkochen, Germany) and an inverted microscope equipped with differential interference contrast (Axiovert S100, Carl Zeiss; with a magnification up to 1000 ×). From each sample, six microscope slides with approximately 500 µL sludge per slide were prepared for live-observation of flocculent

sludge. After formation of granules single granules were selected randomly from the samples and transferred together with 500 µL wastewater liquid into glass petri dishes for the observation of ciliated protists on the granule surface. The succession of protist communities was monitored during the whole granule formation process in SBRD, SBRM, and SBRB with emphasis on ciliates which were the dominating protist taxon. Specimens were identified with special focus on stalked ciliates of the subclass *Peritrichia* which are proven to play an important role in granule structure formation (Weber et al., 2007). Peritrichous ciliates were identified using the guidelines of Foissner et al. (1991, 1992, 1994, 1995). The community composition of ciliates on the surface of granules from SBRS1 and SBRS2 was determined twice monthly. Scanning electron microscopy (SEM) and appropriate sample preparation for freeze fracture analysis were performed as described previously (Huber et al., 1998; Weber et al., 2007).

2.4 Identification of bacteria. The bacterial communities of SBRM, SBRB, SBRS1, and SBRS2 were analyzed performing FISH experiments with fluorescently labeled 16S rRNA-targeted oligonucleotide probes specific for different taxonomic groups (see below). FISH analysis according to the protocol of Weber et al. (2007) was carried out with sludge flocs, homogenized granules, as well as sections and parts of granular biofilms in SBRM and SBRB before and after granule formation, and with granules from SBRS1 and SBRS2.

For the hybridization experiments 46 *Prokarya*- and 3 *Eukarya*-specific rRNA-directed oligonucleotide probes as listed in Tab. 1 were applied. Probes were synthesized and labeled at the 5'-end with the sulfoindocyanine dyes Cy3 or Cy5, or with 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) by MWG (Ebersberg, Germany). Hybridization experiments were performed in a top-down analysis, i.e. probes for hierarchically higher taxa were applied first, and only after confirmed presence of these target organisms in the samples specific probes for bacterial genera or taxonomic groups within these taxa were applied.

Herein, several probes were used for the identification of filamentous bacteria. Concomitantly, Gram and Neisser stains were performed to identify filamentous bacteria as described by Eikelboom and van Buijsen (1999). The EUB probes specific for Bacteria and EUK probes specific for *Eukarya* were applied in all experiments as positive controls. FISH preparations were stained with the DNA-specific dye diamidinophenolindole (DAPI) forming fluorescent complexes with natural double-stranded DNA to visualize prokaryotic cells and the macronuclei of eukaryotes. The FISH-DAPI preparations were investigated with a confocal laser scanning microscope (CLSM; LSM 510, Carl Zeiss) equipped as described previously (Fried et al., 2002).

First, the bacterial composition was checked with all *Prokarya*-specific oligonucleotide probes in homogenized biofilm samples. Thereafter, all probes which were tested positive in the samples, i.e. showing fluorescence signals with their target organisms, were applied in following experiments with granule sections, condensed flocs from phase 2, and parts of granules which contained many treelike colonies of stalked ciliates or fungal hyphae.

FISH analysis on granule sections was performed to (1) identify bacteria attached to the stalks of ciliates and, if present, fungal hyphae on the granule boundaries and (2) to investigate spatial distribution of the bacterial taxa in the biofilm slices. In addition, the granule parts prepared with tweezers from the intact biofilms were used to identify bacteria associated with the ciliate stalks. Moreover, the presence of bacteria in ciliate food vacuoles was observed in the hybridization experiments to evaluate the feeding behavior of stalked ciliates and their possible ecological influence on the wastewater system. Every hybridization experiment of this study was performed three times to verify hybridization results.

The selection of FISH probes for the detection of bacteria in this study is primarily based on published data and some personal communications (Schade, Stöcker, 2007).

3 Results and Discussion

3.1 Diversity of ciliated protists. The SBR set-ups with dairy, malthouse, and brewery wastewater showed a clear succession of ciliate communities, always starting with a mixture of sessile and free swimming organisms. Figures 1 A-C show an overview of protozoan community succession in SBRD, SBRM, and SBRB during the granulation process, respectively. The ciliate community in SBRD included mainly the genera *Vorticella*, *Carchesium*, *Epistylis*, *Opercularia*, *Litonotus*, *Prorodon*, and *Tetrahymena* with *Vorticella microstoma* complex, *Opercularia* sp. and *Tetrahymena* sp. dominating phase 3. In SBRM and SBRB especially the genera *Vorticella*, *Opercularia, Epistylis, Epicarchesium, Litonotus, Acineria, Colpoda,* and *Aspidisca* were found with *Vorticella* spp. and *Opercularia* spp. dominating phase 3;. In SBRB *Opercularia* spp. and *Epistylis* spp. dominating phase 3. In addition *Tokophrya quadripartita* and species of the genus *Pelagothrix* were detected, while *Colpoda* spp. was not found. Fig. 2 represents a selection of peritrichous ciliates found in the SBR samples.

Some ciliates were only temporarily detected in the reactors, e.g. *Uronema* sp., *Urotricha* sp. in SBRD, *Cinetochilium margaritaceum*, *Trochilia minuta*, *Vorticella infusionum*, *Opercularia* spp. in SBRM and *Tokophrya quadripartita* and *Pelagothrix* sp. in SBRB. For some ciliates such as *Opercularia* spp. this may be caused by their ability to turn from active forms into dormancy cysts and back into active cells, depending on environmental changes (Fried, 2002; Blatterer, 2005). Other ciliates may have been brought into the reactors during wastewater exchange but were unable to sustain their population. They either died or were washed out again and were thus only detectable for a short period.

Two weeks after the start of SBRD, representatives of the *Vorticella microstoma* complex and *Opercularia* spp. and the free swimming *Tetrahymena* sp. dominated the ciliate community. *Tetrahymena* sp., which occurred only in SBRD, withstood the wash-out periods by hiding in the caverns of flocs and granules. The ciliate community of SBRM and SBRB was throughout

dominated by attached forms after several weeks, i.e. peritrichous ciliates. All free swimming forms were washed out and could not compete with the attached forms. In all reactors some of the peritrichous ciliates established during granule development and displaced other attached organisms as indicated in Fig. 1. After granule formation only few species, predominantly *Epistylis chrysemydis*, *Epistylis entzii*, *Opercularia asymmetrica* or *Vorticella spp.*, were present in high abundance and colonized the granules in large numbers. During phase 2 of granule formation in SBRB also *Vorticella* spp. vanished while *Opercularia* spp. and especially the number of *Epistylis* specimens proliferated.

The ciliate communities in the steady state reactors SBRS1 and SBRS2 were dominated solely by *Opercularia* spp. and remained stable over the whole monitoring period of four months. The lower ciliate diversity in SBRS 1 and 2 reactors compared to the other reactors might be caused by feeding with onesided artificial wastewater without particulate substrate. Thus, the ciliate diversity originated from the seed sludge composition only and was not influenced by a continuous addition of raw wastewater during reactor operation.

The observation of dominant protozoa in all reactors leads to the conclusion that some ciliate organisms like *Opercularia* spp. and *Epistylis* spp. proved to be very flexible in coping successfully with different types of wastewater. Although *Vorticella* spp. established similarly to *Epistylis* spp. and *Opercularia* spp. in all the different wastewater types, several species of the genus *Vorticella*, i.e. *V. minima* and representatives of the *V. infusionum* complex, *V. microstoma* complex, *V. convallaria* complex, *V. aquadulcis* complex, and *V. octava* complex seem to response more sensitive to environmental changes and could not compete with the latter.

3.2 Diversity of Bacteria. In all samples bacteria were detected generally with the EUB probes (see Tab. 1). Most of the bacteria were rod-shaped, which is a preferred form in biofilms to facilitate cell-to-cell connection and to diminish the impact of fluid shear (Young,

2006). The bacterial community composition, investigated with specific probes as listed in Tab. 1, varied with the various wastewaters of the reactors. Additionally, some taxonomic groups could only be found in phase 1 or in phase 2 within an individual SBR set-up, e.g. in SBRB *Firmicutes* were found only in granules and *Actinobacteria* only in flocculent sludge.

In all SBR samples and in all granulation phases *Alphaproteobacteria* and *Betaproteobacteria* were identified, forming the major part of the activated sludge community, followed by *Gammaproteobacteria* in SBRB and *Actinobacteria* in SBRM, SBRS1, and SBRS2.

- Alphaproteobacteria

Alphaproteobacteria are morphologically extremely diverse (Dworkin & Falkow, 2006). Most of them are rod-shaped and some exhibit filamentous forms, e.g. '*Candidatus* Alysiosphaera europaea' or '*Candidatus* Monilibacter batavus' being readily detected in industrial bulking sludge (Kragelund et al., 2006; Levantesi et al., 2004). However, the application of the probes MC2-649 specific for *C.* Monilibacter batavus and Noli-644 specific for *C.* Alysiosphaera europaea, known to proliferate in brewery and food/potatoe industrial wastewater, respectively (Eikelboom et al., 2002), did not result in positive signals after hybridization in any of the tested reactors. In SBRS2 granules *Alphaproteobacteria* were detected exclusively in the morphology of tetrads (Fig. 3 E, F). Tetrad-forming *Alphaproteobacteria* were already found in enhanced biological phosphorus removal (EBPR) reactors. They have been assumed to represent glycogen-accumulating organisms (GAO) with possible relationship to *Defluviicoccus* sp. according to 16S rRNA analysis (Wong et al., 2004 and 2007; Oehmen et al., 2006). According to de Kreuk & van Loosdrecht (2004) GAOs might enhance granule formation. However, their role in the deterioration of full-scale EBPR processes is not fully clarified.

The presence of *Nitrobacter* sp., a common nitrite oxidizing bacterium (NOB) within the *Alphaproteobacteria*, was checked with the *Nitrobacter*-specific probe NIT3 but no signals were obtained after hybridization. The same was reported in a recent study on activated

sludge by Maixner et al. (2006): No signals were obtained with NIT3 while concurrently the presence of *Nitrospira* spp. was verified in the samples with another probe. This finding is confirmed in this study (see below "*Betaproteobacteria"*).

- *Betaproteobacteria*

Signals of bound probes targeting *Betaproteobacteria* were always related with rod-shaped bacteria, which occasionally occurred in clubbed clusters (Fig. 3 A, B) or as single rods associated with ciliate stalks (Fig. 3H). While specifying the *Betaproteobacteria*, *Sphaerotilus natans*, a filamentous bacterium being readily found in food industry and brewery wastewater (Lemmer & Lind, 2000), was identified to proliferate in SBRB granules.

Specification of betaproteobacterial ammonia oxidizers (AOBs) of the *Nitrosomonas oligotropha* lineage, which are common representatives of the AOBs in the wastewater purification process, was conducted with the probes NEU, Nmo218, and Cluster6a192. After hybridization signals were received exclusively with the probe Cluster6a192 which bound to small spherical cell clusters, tightly packed with short rods. Spherical clusters of rods were also reported previously as a preferred structure formed by AOBs in a study on aquaculture activated sludge (Paungfoo et al., 2007).

No signals were received after hybridization with the probes Nso190 and Nso1225 which theoretically target most of the known AOBs.

For detection of NOBs among the *Betaproteobacteria* the probes ntspa1431, ntspa1151, ntspa662, and Nsv443 were applied to identify *Nitrospira* sp.. Hybridization-derived signals were received after FISH with ntspa1431 in SBRB and SBRS1. Bacteria detected after FISH with ntspa1431 were always rod-shaped. In SBRB samples these rods were arranged in chains (Fig. 4 B) whereas in SBRS1 samples spherical rod-shaped bacterial clusters were found. This implicates that the wastewater type might be decisive for the cluster morphology of these organisms.

- Gammaproteobacteria

Gammaproteobacteria were found in mature granules from SBRS1, SBRB, and in all granulation phases of SBRM. In the SBRM samples they were detected frequently in vacuoles of peritrichous ciliates.

Further specification of the *Gammaproteobacteria* using probe NmV specific for the *Nitrosococcus mobilis* lineage resulted in signals which were hardly distinguishable from the negative control (FISH without addition of a probe) while the EUB probe after FISH showed clear signals. Therefore, the NmV signals were handled as unspecific. This assumption is supported by the concurrent finding that all *Gammaproteobacteria* detected with Gam42a were exclusively rod-shaped and never coccoid as are the nitrosococci in general.

Specifying filamentous *Gammaproteobacteria* showed the sulfur bacterium *Thiothrix nivea* to thrive in SBRB.

- Actinobacteria

Actinobacteria were targeted with both the probe HGC69a which bound to *Actinobacteria* in all SBRs during all granulation phases and the probe HGC1156 showing additionally clear positive signals in SBRB in phase 1. *Actinobacteria* occurred in large numbers in SBRS1 as discoidal cells arranged in short chains (Fig. 3 C, D) resembling *Nostocoida limicola* morphotypes. In wastewater treatment without microbial granules these organisms are known to cause sludge foaming and bulking (Schade et al., 2002). However, implemented in granules they did not induce such problems. Further specification of other filamentous *N. limicola* morphotypes was performed by applying the probes NLII65 and NLIMII 175 both targeting the *N. limicola* II morphotype as well as NLIMI 91 targeting the *N. limicola* I morphotype. Signals were derived only with NLIMI 91 from single coccoid bacteria. Since the probe NLIMI 91 concurrently is specific for *Streptococcus* sp. it is to be supposed, that this organism was present in the samples. This correlates also with the fact that *N. limicola* I chains consist of different bacterial genera including *Streptococcus* sp. (Liu et al., 2000).

Nevertheless, two other probes for coccoid bacteria were tested to exclude false positive results: Efi58 and Efe1463 are both specific for *Enterococcus* sp., an organism which often occurs in activated sludge. No hybridization signals were received with these probes with currently strong signals from positive controls hybridized with the EUB probe mix.

In SBRB *Actinobacteria* were apparent in the form of short rods. In a previous study on wastewater treatment short rods were the most abundant form of *Actinobacteria* besides tetrad-arranged cocci (Kong et al., 2005).

In summary, sludge samples comprised a high bacterial diversity, which differed between the SBR samples as described in this section and also listed in Tab. 1. However, just as granulation has previously been found not to be a function of specific microbiological groups (Beun et al., 1999; Wang et al., 2004) also in this study the bacterial composition of the granules seemed not to follow specific rules.

3.3 Filamentous bacteria in SBRB. Filamentous bacteria generally are undesired in wastewater treatment, since they can cause sludge bulking and foaming problems (Eikelboom, 2000; Jenkins et al., 2004; Tandoi et al., 2006). In SBRB the presence of these organisms led to the formation of extremely large granules up to 6 mm in diameter. Their structure was less compact as compared to the biofilms from the other reactors observed. The fluffy structure dramatically decreased the settling velocity as is also reported by Zheng et al. (2006). We called these biofilms 'bulking granules'. As described previously (Gaval et al., 2002; Weber et al., 2007) the occurrence of bacterial filaments in SBRB might be addressed as a stress reaction due to temporary oxygen deficiencies as a result of high temperatures up to 35°C during summer time plant operation. Furthermore, a COD loading rate of 3.6 kg m^{-3} d^{-1} being significantly higher than that of the other reactors and bearing a high percentage of carbonaceous load, might have led to nutrient limitations, i.e. deficiencies in N and P. This is

a prevalent cause of formation of bacterial filaments. They are assumed to increase their total surface area to get preferred access to solved nutrients (Young, 2006). Several probes were used to identify these filaments. After hybridization with TNI, SNA, and G123T different filamentous bacteria showed clear fluorescence signals (Tab. 1). The identification results were consistent with Gram and Neisser reactions of the tested filaments. Thus, the presence of *Sphaerotilus natans*, *Thiothrix* sp., and *Thiothrix nivea* was proven for SBRB samples. *Sphaerotilus natans* and *Thiothrix nivea* were also detected in the same hybridization experiment with group-specific FLUOS-labeled probes targeting *Betaproteobacteria* and *Gammaproteobacetria,* respectively. Additionally, the typical formation of rosettes as indicated for *Thiothrix* sp. (Eikelboom, 2000) was found. The presence of these filaments in the SBRB is not surprising as *Sphaerotilus natans* is well known to proliferate at high load conditions with readily degradable food such as sugars and short chain fatty acids with N and/or P deficiency. Furthermore, the sulfur bacteria are readily coping with oxygen deficiency conditions (Lemmer & Lind, 2000).

3.4 Spatial distribution of bacteria and protists in the biofilm. Living ciliates were found on the surface and in caverns of granules constituting the fringe zone of the biofilms. Furthermore, the outer surface occasionally showed protruding fungal hyphae and filaments as is also reported for brewery wastewater in a previous study (Wang et al., 2007; Weber et al., 2009). The area directly beneath the granules' surface exhibited remnants of ciliate stalks and sometimes dead ciliate cell bodies while inner core zones of granules consisted of bacteria only (Weber et al., 2007). Hybridization analysis of granule sections showed no spatial preferences of the identified bacteria within the biofilms. Granules did not exhibit specific bacterial distribution layers and the detected taxa mostly arranged in clusters, were homogenously distributed over the whole granule. A non-layered microbial distribution in aerobic granules was also reported by Wang et al. (2004). However, some other studies on

aerobic (Tay et al., 2003) and anaerobic granules, e.g. from EBPR reactors (Batstone et al., 2004; Abreu et al., 2007), reported specific patterns detected throughout the granule depending on different metabolic activities and on substrate availabilities of the bacterial species in the biofilm. The more or less homogenous distribution of bacteria in our granules might be due to a balanced nutrient supply throughout the biofilm by virtue of channels, voids, and pores being typical for aerobic granules. This is confirmed by previous studies of Tay et al. (2003) and Wang et al. (2004) for aerobic granules with diameters up to 8 mm. Furthermore, the cilia beat of peritrichous ciliates creates flow fields up to 200 µm from the location of the cell, as reported from Hartmann et al. (2007) for *Opercularia asymmetrica*. This flow field supports the nutrient flux towards, into, and within the biofilm (Hartmann et al., 2007; Fried & Lemmer, 2003).

3.5 Association of bacteria and ciliates. Light microscopy and scanning electron micrographs showed that bacteria used ciliate stalks or, if present, fungal hyphae as substratum to grow. They were associated with the eukaryotes in large numbers. Throughout the investigations only rod-shaped cells affiliated with various phyla were shown to be associated with ciliate stalks. Short rods abutted lengthwise along the stalks and long rods protruded orthogonally grabbing hold of the stalks and hyphae with their pili. Examining the micrographs (Fig. 3G, 5C) the question arises if this association is coincidentally or a matter of a species- or morphology-based specific relationship. Hybridization experiments with the universal *Bacteria*-specific EUB probes always resulted in clear and strong signals from bacteria and ciliates (Fig 5). After further FISH analyses bacteria associated with ciliate stalks were mainly assigned to rod-shaped *Gammaproteobacteria* and *Betaproteobacteria* (Fig. 3H). In SBRB, *Nitrospira* sp. were detected among the *Betaproteobacteria* which may have attached initially to the stalks as single cells and, afterwards, formed chains (Fig. 4B). In SBRB also short rods of the *Actinobacteria* tightly attached to the stalks were detected (Fig.

4A). Short rods have more contact points than spherical-shaped bacteria and long rods often exhibit a large number of pili on the cell poles. Thus they might stick better to stalks or hyphae than otherwise shaped bacteria. Since in our case only rods were found attached to ciliate stalks this is an additional indication for the observations of Young (2006) that cell shape seems to be more decisive for attachment than specific bacterial taxa.

3.6 Competition between bacteria and ciliates

3.6.1 Biofilm formation. This is a strategy of microorganisms to protect themselves against harmful environmental influences. Nevertheless, inside the biofilms there is a struggle for survival. In SBRB the enormous proliferation of filamentous bacteria resulted in a strong competition between bacteria and ciliates on the granule surface: (1) Due to their growing pattern filamentous bacteria protrude from the granule surface as do peritrichous ciliates. Thus, ciliates compete with filaments for a settling site on the granule surface. (2) Since many filamentous bacteria are growing faster than ciliates they might overgrow the ciliates. Thereby, the filaments enwrap the ciliate cells and hamper their cilia beat. This on the one hand causes a permanent dissolution of parts of the peritrichous ciliate colonies (Weber et al., 2007). On the other hand it results in the gain of new substratum for bacterial growth. (3) Consequently, competition between bacterial filaments and ciliates for substratum to grow on will also result in a higher selection pressure within the peritrichous ciliates. Fast growing ciliates will outcompete slow growers. For example in SBRB, the fast growing *Epistylis chrysemydis* finally dominated the granules surfaces and outnumbered *Epistylis entzii* and *Opercularia asymmetrica*.

3.6.2 The predator-prey relationship between ciliates and bacteria. Detaching bacteria together with organic particles and dissolved nutrients are the main food supply for ciliates in granular biofilms. Thus, a focus was set on the determination of the predominant bacteria being present in ciliate food vacuoles. Generally, no differences between the bacterial composition in the biofilm as compared to the selection in vacuoles were found. Solely SBRM biofilms showed predominantly *Gammaproteobacteria* to be present in the vacuoles. There are two possible explanations for this finding: First of all, more *Gammaproteobacteria* were found in the SBRM granules than in any other SBR biofilm and therefore, their occurrence in the vacuoles likewise was significantly higher. Secondly, most of the

Gammaproteobacteria did not occur in tightly packed clusters, as was found in this study for *Betaproteobacteria* and *Alphaproteobacteria.* In SBRM they did not form filaments either as it was the case for *Thiothrix* sp. in SBRB. Thus, beside barley grains these bacterial single cells might be an easily accessible nutrient supply for ciliates. As they were generally rod-shaped easy accessibility seems again to depend more on shape than on phylogeny.

In SBRB biofilms many filamentous bacteria were found. The possible selection of filaments by temporary oxygen deficiency and nutrient limitation was stated above. Another reason for filament formation might have been to escape ciliate predation (Shikano et al., 1990; Jürgens et al., 1991; Jürgens & Matz, 2002; Matz et al., 2006). Since remarkably high numbers of ciliates were found in these granules they might have created an additional selective pressure on the bacterial biocenosis and have supported filamentous growth.

Actinobacteria were never found in any ciliate vacuole in any granule biofilm. This could be due to ciliates preferences as they are described to mostly select for Gram negative bacteria, because they might be easier to digest than Gram positives (Gonzalez et al., 1990; Iriberri et al., 1994; Pernthaler J., 2005).

4 Conclusions

Our study suggests some general rules to exist for the interaction of prokaryotes and eukaryotes during the development of aerobic granules:

- It is obvious that a defined process in the population dynamics of ciliates takes place during granular biofilm formation in all approaches: A high diversity of ciliates in the flocculent seed sludge is always followed by establishment of specific peritrichous ciliate taxa during granule formation.

- The diversity of ciliates seems to be influenced by a variety of selection factors such as operation parameters or wastewater composition.

- The selection of peritrichous ciliate species might be influenced by the presence of bacterial filaments, competing for settling sites on the biofilm surface.
- Bacteria always use the stalks of ciliates as substratum to grow. The physical shape of bacteria might thereby play an important role.
- Analyses in this study based on whole cell FISH with rRNA-directed probes support the shape-based relationship hypothesis since associated bacteria were exclusively rod-shaped.
- Bacterial diversity in granules seems to depend on wastewater composition. However, in terms of spatial arrangement the bacterial taxa within the biofilm are shown to be homogenously and randomly distributed.

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Figure legends

Fig. 1: Protist community analysis of sequencing batch reactors (SBR). Succession of ciliates and flagellates with regard to phases of granules development. Abundance of organisms within 500 µL: – not present; ▉ few cells present; ▉▉ several cells present; ▉▉▉ many cells present. Granules development: Phase 1, mixed community of diverse free swimming protozoa and small stalked colonies of ciliates on activated sludge flocs; Phase 2, dense flocs are formed with large colonies of peritrichous ciliates and bacteria accumulating in the center of the flocs; Phase 3, granules are formed, diversity of ciliates is reduced to a few very abundant species which settle in large numbers on the surface of biofilms. **A:** SBRD, dairy wastewater. **B:** SBRM, malthouse wastewater. **C:** SBRB, brewery wastewater.

Fig. 2: Dominant peritrichous ciliates in the different sequencing batch reactors (SBR). **A, D:** *Epistylis chrysemidis*; **B, E:** *Vorticella* sp. **C:** *Opercularia* sp.; **F:** Example of an only temporarily detectable ciliate in SBRB (brewery wastewater) during phase 1 and 2 of granule development: *Tokophrya quadripartita*.

Fig. 3: Selection of various bacterial morphotypes present in the sequencing batch reactors (SBR) illustrated with scanning electron micrographs (SEM) and probe-related signals of appropriate morphological forms from same samples after FISH recorded by CLSM. (fluorescein-labeled probes: green, CY3-labeled probes: red; DAPI staining after FISH: blue). Clubbed clusters of short rods (A) detected also with the *Betaproteobacteria*-specific probe Bet42a-Cy3 in SBRS1 (synthetic wastewater, flow rate of reactor 4 L min^{-1}) (B), discoidal bacteria arranged in chains (C) with the *Actinobacteria*-specific probe HGC69a-FLUOS in SBRS1 samples (D), and tetrad-arranged bacteria (E) with the *Alphaproteobacteria*-specific probe Alf968-Cy3 in SBRS2 (synthetic wastewater, flow rate of reactor 6 L min^{-1}) (F). Short and long rod-shaped bacteria grabbing hold with their pili lengthwise the ciliate stalk or protruding orthogonally (G). Long rods were also detected with the *Betaproteobacteria*-specific probe Bet42a-Cy3 in SBRB (brewery wastewater) (H).

Fig. 4: Probe-related signals after FISH in sequencing batch reactor SBRB (brewery wastewater). Small rods (A) and rods in chains (B) are both attached to peritrichous ciliate stalks. Probe-related signals (Cy3, red) and DAPI staining (blue) are recorded by CLSM after FISH with the *Actinobacteria*-specific probe HGC69a (A) and the *Nitrospira*-specific probe ntspa1431 (B).

Fig. 5: Probe-related signals after FISH applied on a treelike ciliate colony on the surface of a single granule developed in malthouse-derived wastewater. The image is recorded by CLSM after FISH with the *Bacteria*-specific mix of the EUB338 probes (fluorescein-labeled; green) and the *Eukarya*-specific probe EUK516 (Cy3-labeled; red) and shows protist cell bodies surrounded by bacteria which are attached to the ciliate stalks. White arrows indicate the location of ciliate stalks, since stalks are lacking of rRNA and comprise no probe target (A). Ciliate cells embedded in bacteria (B) and ciliate stalks with attached bacterial rods (C) illustrated with scanning electron micrographs.

Figure 1A

SBRB \ Phase	1						2						3				
Species \ Day	**1**	**8**	**9**	**11**	**15**	**17**	**21**	**22**	**24**	**35**	**38**	**43**	**56**	**59**	**63**	**66**	**77**
Vorticella spp.	■	■	■	■	■	■	■	■	■	-	-	-	-	-	-	-	-
Vorticella infusionum complex	■	■	■	■	■	■	■	-	-	-	-	-	-	-	-	-	-
Vorticella microstoma complex	-	■	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-
Vorticella convallaria complex	■	■	■	-	-	-	■	-	-	-	-	-	-	-	-	-	-
Vorticella minima	■	-	■	-	-	-	■	-	-	-	-	-	-	-	-	-	-
Vorticella aquadulcis complex	■	-	■	-	-	-	■	-	-	-	-	-	-	-	-	-	-
Vorticella octava complex	■	■	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-
Opercularia spp.	■	-	-	-	-	■	■	■	■	■	■	■	■	■	■	-	■
Opercularia assymetrica	-	-	-	-	-	■	■	■	■	■	■	■	■	■	■	-	■
Opercularia coarctata	-	-	-	-	-	-	■	-	-	-	-	-	-	-	-	-	-
Epistylis spp.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Epistylis chrysemidis	-	■	-	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Epistylis entzii	-	-	■	■	■	■	■	■	■	■	■	■	■	■	■	-	-
Epistylis hentscheli	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epistylis coronata	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Zoothamnium sp.	-	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	-
Tokophrya quadripartita	-	■	■	-	-	-	-	-	■	-	-	-	-	-	-	-	-
Aspidisca cicada	■	■	■	■	-	-	-	-	-	-	-	-	-	-	■	-	-
Acineria sp.	■	■	■	-	-	-	■	■	■	-	■	■	■	-	-	-	-
Colpoda sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	■
Pelagothrix sp.	■	-	-	-	-	-	-	■	-	-	-	-	-	-	-	-	-
Suctoria	-	■	■	-	-	-	■	-	■	-	-	-	-	-	-	-	-
Flagellates	■	-	■	-	-	-	-	■	-	-	■	-	-	-	-	-	-

Figure 1B

SBRD \ Phase	1					2						3					
Species \ Day	**1**	**2**	**3**	**4**	**5**	**8**	**9**	**10**	**12**	**15**	**17**	**19**	**23**	**25**	**31**	**33**	**37**
Vorticella infusionum complex	■	■	■	■	■	■	■	■	■	-	-	-	-	-	-	-	-
Vorticella microstoma complex	■	■	■	■	-	-	-	-	-	-	■	■	■	■	■	■	■
Vorticella convallaria complex	-	-	-	■	-	■	-	-	-	-	-	-	-	-	-	-	-
Opercularia spp.	■	-	-	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Epistylis spp.	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epicarchesium sp.	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carchesium sp.	■	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Litonotus sp.	■	■	■	■	■	-	-	■	■	-	-	-	-	-	-	-	-
Aspidisca cicada	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Prorodon sp.	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pelagothrix sp.	-	-	-	■	■	-	-	-	-	-	-	-	-	-	-	-	-
Tetrahymena sp.	-	-	-	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Uronema sp.	-	-	-	-	-	-	-	-	■	■	-	-	-	-	-	-	-
Urotricha sp.	-	-	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Suctoria	-	-	-	-	-	-	-	-	-	■	■	■	-	-	-	-	-
Flagellates	-	-	-	-	-	-	-	-	-	■	■	-	-	-	-	-	-

Figure 1C

SBRM \ Phase	1				2			3														
Species \ Day	**1**	**3**	**10**	**15**	**17**	**21**	**28**	**41**	**44**	**48**	**63**	**66**	**70**	**73**	**91**	**94**	**98**	**99**	**102**	**122**	**127**
Vorticella spp.	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
Vorticella infusionum complex	■	■	-	-	-	-	-	-	-	■	-	-	-	-	-	-	-	■	-	-	-
Vorticella microstoma complex	■	■	■	■	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Vorticella convallaria complex	■	■	■	■	■	■	-	-	■	■	-	-	-	-	-	-	-	-	-	-	-
Vorticella elongata	■	■	■	■	■	■	-	-			-	-	-	-	-	-	-	-	-	-	-
Opercularia spp.	■	■	■	■	■	■	■	-	■	■	■	-	-	-	■	-	-	-	-	-	-
Opercularia assymetrica	■	■	■	■	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epistylis spp.	■	■	■	■	■	■	■	■	■	■	■	■	-	■	■	■	■	■	■	■	■
Epistylis chrysemidis	■	■	-	-	-	-	-	-	-	-	■	-	-	-	■	■	■	■	■	■	■
Epistylis coronata	-	-	■	■	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epistylis procumbens	-	-	■	■	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epicarchesium sp.	■	■	■	■	■	■	-	■	■	-	-	-	-	-	-	-	-	■	-	-	-
Carchesium sp.	■	■	■	■	■	-	-	■	■	-	-	-	-	-	-	-	-	■	-	-	-
Zoothamnium sp.	■	■	-	-	-	-	-	■	■	-	-	-	-	-	-	-	-	■	-	-	-
Litonotus sp.	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspidisca cicada	■	■	■	■	■	-	■	-	■	-	■	-	-	-	-	-	-	-	-	-	-
Acineria sp.	■	■	-	-	-	-	-	■	■	■	■	-	-	-	-	-	-	-	-	-	-
Colpoda sp.	■	■	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trochilia minuta	-	-	-	-	-	-	-	-	-	■	■	■	■	-	-	-	-	-	-	■	■
Cinetochilium margaritaceum	-	-	-	-	-	-	-	-	-	■	■	■	■	-	-	-	-	-	-	-	-
Flagellates	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	-	-	-	-	-

Figure 2

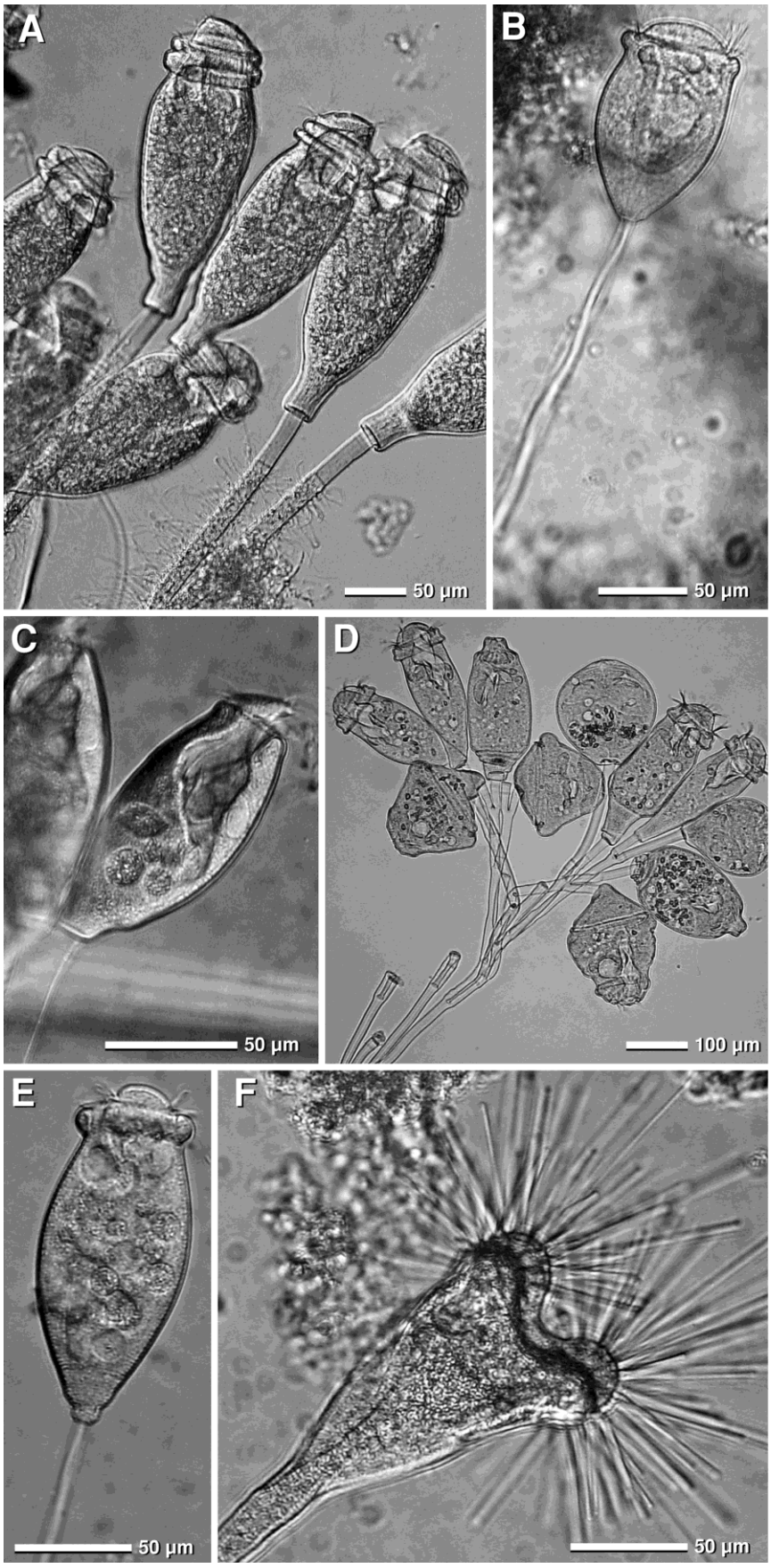


Figure 3

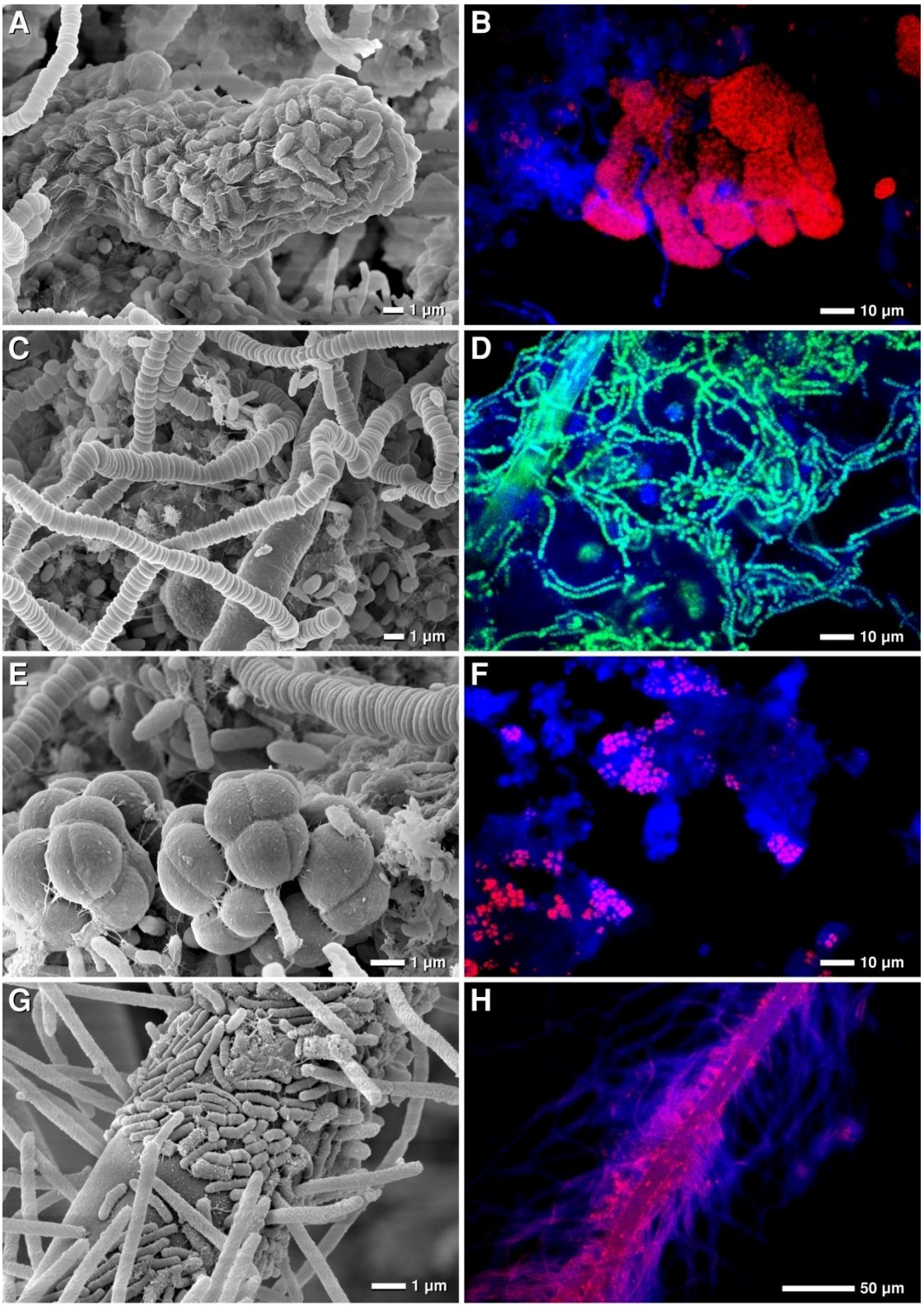


Figure 4

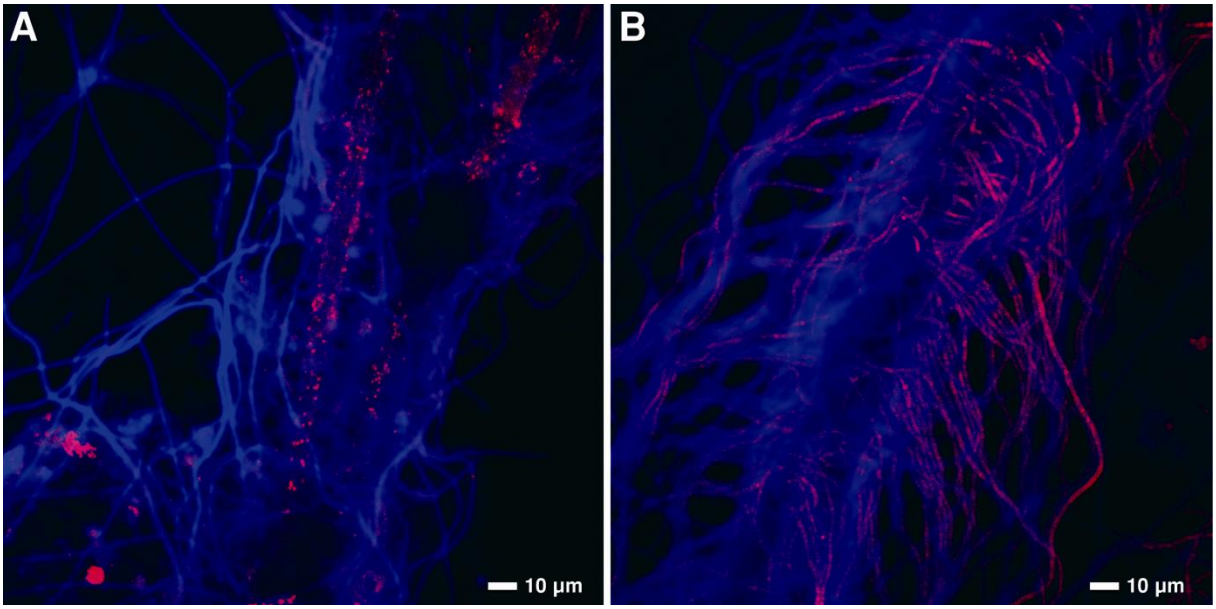
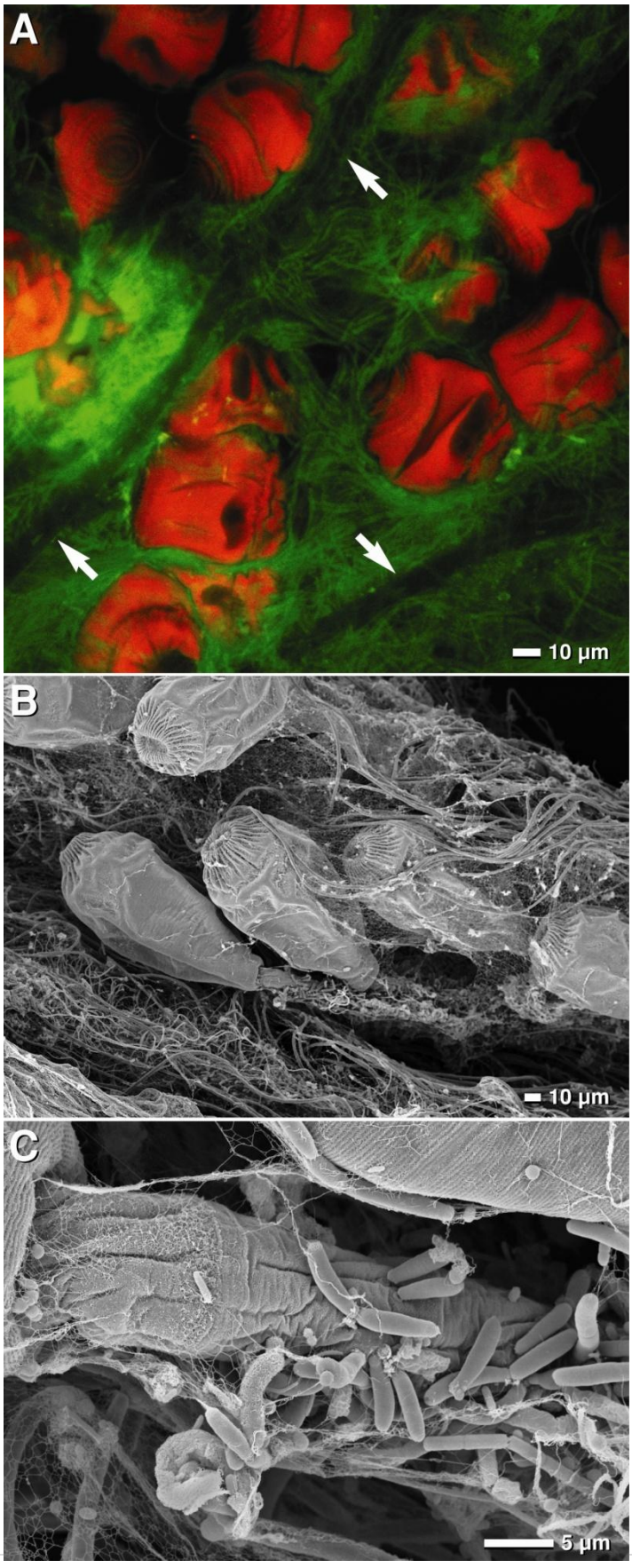


Figure 5



Tab. 1: FISH results with different oligonucleotide probes (probe details listed in Tab. 2, see supplementary material) and with samples from different sequencing batch reactors (SBR) operated with malthouse (SBRM), brewery (SBRB), and synthetic wastewater with flow rate 4 L min^{-1} (SBRS1) and 6 L min^{-1} (SBRS2) .. Presence of bacterial taxa in flocculent seed sludge and granules is shown. + = present, o = presence not verified due to unclear signals, - = not present.

Probe name		SBRM		SBRB		SBRS1	SBRS2
		floc	granule	floc	granule	granule	Granule
EUB338	Amann et al., 1990	+	+	+	+	+	+
EUB338 II	Daim et al., 1999	+	+	+	+	+	+
EUB338 III	Daim et al., 1999	+	+	+	+	+	+
EUK516	Amann et al., 1990	+	+	+	+	+	+
EUK502-1	Fried, 2004	+	+	+	+	+	+
EUK1195	Giovannoni et al., 1988	+	+	+	+	+	+
Bet42a	Manz et al.,1992	+	+	+	+	+	+
Gam42a	Manz et al.,1992	-	-	+	+	+	-
Alf1b	Manz et al.,1992	+	+	+	+	+	+
Alf968	Neef A., 1997.	+	+	-	-	+	+
Delta495a,b,c Mix	Loy et al., 2002	-	-	-	-	+	+
Epsy549	Lin et al., 2006	o	+	+	+	+	O
Epsy1384	Loy et al., 2007	+	+	+	+	+	O
LGC354A,B,C Mix	Meier et al., 1999	o	o	-	+	+	+
HGC69a	Roller et al., 1994	+	+	+	-	+	+
HGC1156	Erhart, 1997	+	+	+	+	+	+
Arc344	Raskin et al., 1994	-	-	-	-	-	-
Arc915	Stahl etal., 1991	-	-	-	-	-	-
nso190	Mobarry et al., 1996	-	-	-	-	-	-
nso1225	Mobarry et al., 1996	-	-	-	-	-	-
nmo218	Gieseke et al., 2001	-	-	-	-	-	-
NmV	Pommerening-Roeser et al.,1996, Juretschko et al., 1998	-	-	-	-	-	-
cf319	Manz et al., 1996	-	-	-	-	-	-
gnsb-941	Gich et al., 2001	-	-	-	-	o	-
cfx-Mix (CFX1223+gnsb-941)	Björnsson et al., 2002	o	-	o	-	-	-
Bone + Comp.btwo	Amann et al., 1996	-	-	-	-	-	-
CHL 1851	Beer et al., 2002	-	-	-	o	-	-
SNA + Comp.CTE23a	Wagner et al 1994a	-	-	+	+	-	-
CTE(23a)	Schleifer et al., 1992	-	-	-	-	-	-

Probe name		SBRM		SBRB		SBRS1	SBRS2
		floc	granule	floc	granule	granule	granule
ACA23a (652)	Wagner et al 1994a	-	-	-	-	-	-
TNI	Wagner et al 1994a	-	-	-	+	-	-
21N	Wagner et al 1994a	-	-	-	-	-	-
G123T + Comp.G123T	Kanagawa et al., 2000	-	-	-	+	-	-
MNP1	Schuppler et al., 1998	-	-	-	-	-	-
NLIMI 91	Liu und Seviour, 2001	+	+	+	-	-	-
NLII65	Bradford D., 1997	-	-	-	-	-	-
NLIMII 175	Liu und Seviour, 2001	-	-	o	o	-	-
Noli-644	Snaidr et al., 2002, Levantesi et al., 2004	-	-	-	-	-	-
PPx3-1428	Snaidr et al., 2002, Levantesi et al., 2004	-	-	-	-	-	-
MC2-649	Snaidr et al., 2002, Levantesi et al., 2004	-	-	-	-	-	-
ntspa1431	Maixner et al., 2006	o	-	+	+	+	
ntspa1151	Maixner et al., 2006	o	-	-	-	-	
ntspa662 +comp.	Daims et al., 2001	-	-	-	-	o	
Cluster 6a192 +comp.	Adamczyk et al. 2003	-	-	+	-	+	
NIT3 +comp.	Wagner et al., 1996	-	-	-	-	-	-
NEU+comp.	Wagner et al., 1995	-	-	-	-	-	-
Nsv443	Mobarry et al., 1996	-	-	-	-	-	-
Efi58	Behr et al., 2000	o	-	-	o	o	o
Efe1473	Ludwig and Santos, 2007	-	-	-	-	-	-

Supplementary Material to Tab. 1 – Oligonucleotide probes used for FISH analysis including indication of probe sequence and reference.

Probe name	Target organism	5′-sequence-3′of probe	Reference
EUB338	most bacteria	GCTGCCTCCCGTAGGAGT	Amann R. I., Binder B. J., Olson R. J., Chisholm S. W., Devereux R. and Stahl D. A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919-1925.
EUB338 II	*Planctomycetales*	GCAGCCACCCGTAGGTGT	Daims H., Brühl A., Amann R., Schleifer K.-H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22: 434-444.
EUB338 III	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	Daims H., Brühl A., Amann R., Schleifer K.-H. and Wagner M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22: 434-444.
EUK516	most eukarya	ACCAGACTTGCCCTCC	Amann R. I., Binder B. J., Olson R. J., Chisholm S. W., Devereux R. and Stahl D. A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing
EUK502-1	most eukarya	ACCAGACTTGCCCTC	by Fried J. 2004, personal communication
EUK1195	most eukarya	GGGCATCACAGACCTG	Giovannoni S. J., DeLong E. F., Olsen G. J. and Pace N. R. (1988). Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. 170: 720-726
Bet42a + Comp.Gam42a	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	Manz W., Amann R., Ludwig W., Wagner M. and Schleifer K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol.15: 593 - 600.
Gam42a + Comp.Bet42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	Manz W., Amann R., Ludwig W., Wagner M. and Schleifer K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol.15: 593 - 600.
Alf1b	*Alphaproteobacteria*	CGTTCGYTCTGAGCCAG	Manz W., Amann R., Ludwig W., Wagner M. and Schleifer K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol.15: 593 - 600.

Alf968	*Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	Neef, A. 1997. Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Dissertation, Technische Universität München, Germany.
Delta495a	*Deltaproteobacteria* and most *Gemmatimonadetes*	AGTTAGCCGGTGCTTCCT	Loy A., Lehner A., Lee N., Adamczyk J., Meier H., Ernst J., Schleifer K.-H. and Wagner M. (2002). Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. 68: 5064-5081.
Delta495b	Some *Deltaproteobacteria*	AGTTAGCCGGCGCTTCCT	Loy A., Lehner A., Lee N., Adamczyk J., Meier H., Ernst J., Schleifer K.-H. and Wagner M. (2002). Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. 68: 5064-5081.
Delta495c	Some *Deltaproteobacteria*	AATTAGCCGGTGCTTCCT	Loy A., Lehner A., Lee N., Adamczyk J., Meier H., Ernst J., Schleifer K.-H. and Wagner M. (2002). Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl. Environ. Microbiol. 68: 5064-5081.
Eprot549/ Epsy549	*Epsilonproteobacteria*	CAGTGATTCCGAGTAACG	Lin, X., Wakeham, S.G., Putnam, I.F., Astor, Y.M., Scranton, M.I., Chistoserdov, A.Y., and Taylor, G.T. 2006. Comparison of vertical distributions of prokaryotic assemblages in the anoxic Cariaco Basin and Black Sea by use of fluorescence in situ hybridization. Appl. Environ. Microbiol. 72: 2679-90.
Eprot1384/Epsy1384	*Epsilonproteobacteria*	CGGTGAGTACAAGACCCG	Loy A, Maixner F, Wagner M, Horn M. 2007. probeBase - an online resource for rRNA-targeted oligonucleotide probes: new features Nucleic Acids Res. 35: 800-804.
LGC354A	*Firmicutes*	TGGAAGATTCCCTACTGC	Meier H., Amann R., Ludwig W. and Schleifer K.-H. (1999). Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. 22: 186-196.
LGC354B	*Firmicutes*	CGGAAGATTCCCTACTGC	Meier H., Amann R., Ludwig W. and Schleifer K.-H. (1999). Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. 22: 186-196.
LGC354C	*Firmicutes*	CCGAAGATTCCCTACTGC	Meier H., Amann R., Ludwig W. and Schleifer K.-H. (1999). Specific oligonucleotide probes for in situ detection of a major group of gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. 22: 186-196.
HGC69a	*Actinobacteria*	TATAGTTACCACCGCCGT	Roller, C., Wagner, M., Amann, R., Ludwig, W. and Schleifer, K.H. (1994) In situ probing of Gram-positive bacteria with high DNA G+C content using 23S rRNA-targeted oligonucleotides. Microbiology 140(10), 2849-2858.

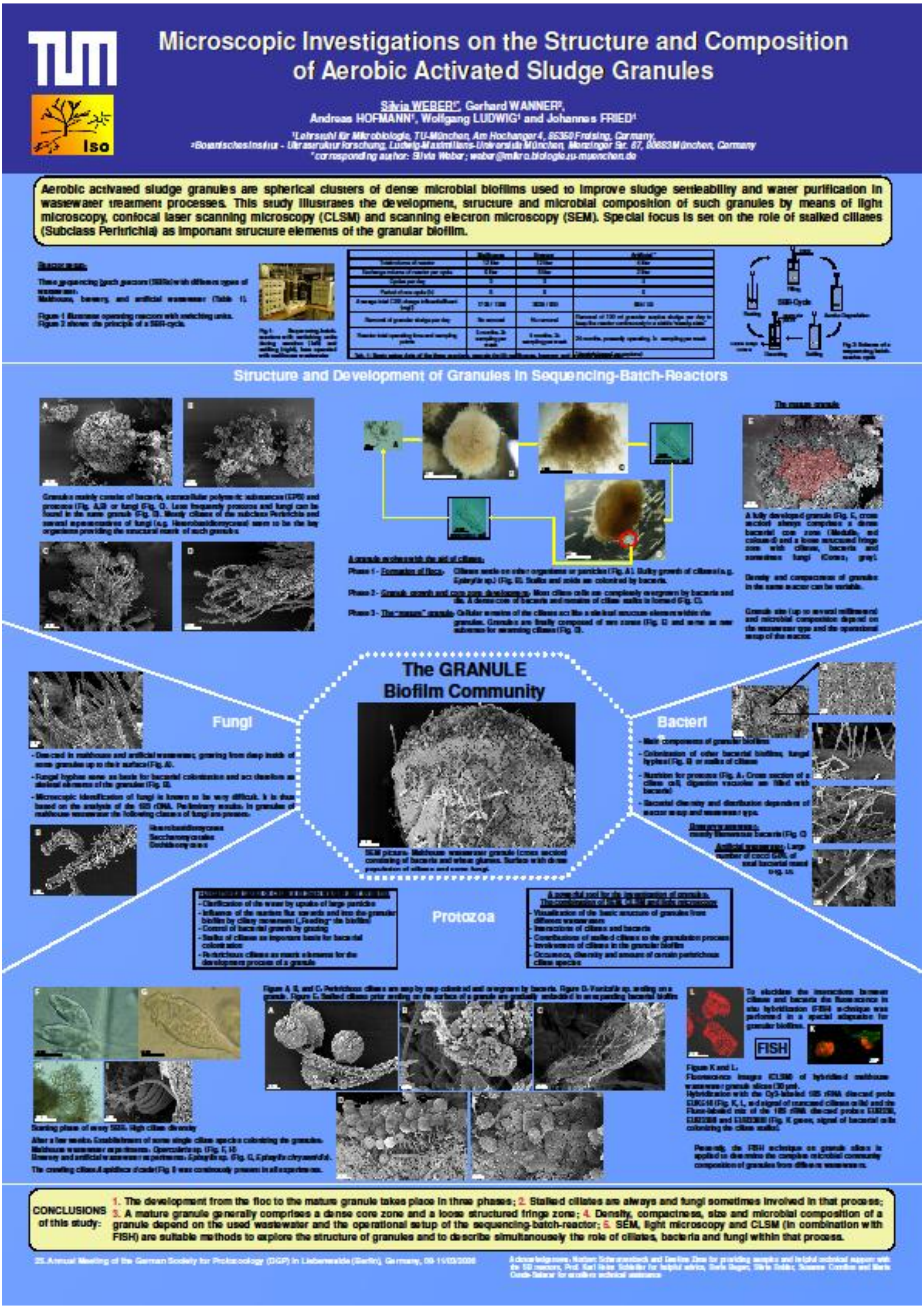
HGC1156	subpopul. HGC group	CGAGTTGACCCCGGCAGT	Erhart, R. (1997) In situ Analyse mikrobieller Biozönosen in Abwasserreinigungsanlagen. Dissertation, Technische Universität München, Germany.
Arc344	*Archaea*	TCGCGCCTGCTGCICCCCGT	Raskin L., Stromley J. M., Rittmann B. E. and Stahl D. A. (1994). Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl. Environ. Microbiol. 60: 1232-1240.
Arc915	*Archaea*	GTGCTCCCCCGCCAATTCCT	Stahl, D. A. and R. Amann. (1991). Development and application of nucleic acid probes. 205-248. In E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons Ltd., Chichester, England.
nso190	Betaproteobacterial ammonia-oxidizing bacteria	CGATCCCCTGCTTTTCTCC	Mobarry B. K., Wagner M., Urbain V., Rittmann B. E. and Stahl D. A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 62: 2156-2162.
nso1225	Betaproteobacterial ammonia-oxidizing bacteria	CGCCATTGTATTACGTGTGA	Mobarry B. K., Wagner M., Urbain V., Rittmann B. E. and Stahl D. A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 62: 2156-2162.
nmo218	*Nitrosomonas oligotropha*-lineage	CGGCCGCTCCAAAAGCAT	Gieseke, A., U. Purkhold, M. Wagner, R. Amann, and A. Schramm. 2001. Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Appl. Environ. Microbiol. 67:1351–1362.
NmV	*Nitrosococcus mobilis* ("*Nitrosomonas*") lineage	TCCTCAGAGACTACGCGG	Pommerening-Roeser, A., Rath, G., and Koops H.-P. 1996. Phylogenetic diversity within the genus Nitrosomonas. System. Appl. Microbiol.19: 344-351. Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Röser, A., Koops, H.P. and Wagner, M. (1998) Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira- like bacteria as dominant populations. Appl. Environ. Microbiol. 64(8), 3042-3051.
cf319	*Cytophaga-Flavobacterium*-group of the *Bacteroidetes*	TGGTCCGTGTCTCAGTAC	Manz W., Amann R., Ludwig W., Vancanneyt M. and Schleifer K.-H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiol. 142: 1097-1106.
gnsb-941	phylum *Chloroflexi* (green nonsulfur bacteria)	AAACCACACGCTCCGCT	Gich F., Garcia-Gil J. and Overmann J. (2001). Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Microbiol. 177: 1-10.

CFX1223	phylum *Chloroflexi* (green nonsulfur bacteria)	CCATTGTAGCGTGTGTGTMG	Björnsson L., Hugenholtz P., Tyson G. W. and Blackall L. L. (2002). Filamentous Chloroflexi (green non-sulfur bacteria) are abundant in wastewater treatment processes with biological nutrient removal. Microbiology. 148: 2309-2318.
Bone + Comp.btwo	beta1-group of *Betaproteobacteria*	GAATTCCACCCCCCTCT	Amann R., Snaidr J., Wagner M., Ludwig W. and Schleifer K. H. (1996). In situ visualization of high genetic diversity in a natural microbial community. J. Bacteriol. 178, 3496-3500
CHL 1851	filamentous bacterium Eikelboom Type 1851	AATTCCACGAACCTCTGCCA	Beer M., Seviour E., Kong Y., Cunningham M., Blackall L. and Seviour R. (2002). Phylogeny of the filamentous bacterium Eikelboom Type 1851, and design and application of a 16S rRNA targeted oligonucleotide probe for its fluorescence in situ identification in activated sludge. FEMS Microbiol. Lett. 207: 179-183.
SNA + Comp.CTE23a	*Sphaerotilus natans*	CATCCCCCTCTACCGTAC	Wagner, M., Assmus, B., Hartmann, A., Hutzler, P. and Amann, R. 1994. In situ analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal scanning laser microscopy. Journal of Microscopy (Oxford) 176(3), 181-187.
CTE(23a)	*Comamonas* spp., *Acidovorax* spp., Hydrogenophaga spp., Aquaspirillum spp.	CATCCCCCTCTACCGTAC	Schleifer K.-H., Amann R., Ludwig W., Rothemund C., Springer N. and Dorn S. (1992). Nucleic acid probes for the identification and in situ detection of pseudomonads. pp. 127-134. In: Pseudomonas: Molecular Biology and Biotechnology. Edited by Galli, E., Silver, S. and Witholt, B. Washington. American Society for Microbiology.
ACA23a (652)	*Acinetobacter*	ATCCTCTCCCATACTCTA	Wagner, M., Assmus, B., Hartmann, A., Hutzler, P. and Amann, R. 1994. In situ analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal scanning laser microscopy. Journal of Microscopy (Oxford) 176(3), 181-187.
TNI	*Thiothrix nivea*	CTCCTCTCCCACATTCTA	Wagner, M., Assmus, B., Hartmann, A., Hutzler, P. and Amann, R. 1994. In situ analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal scanning laser microscopy. Journal of Microscopy (Oxford) 176(3), 181-187.
21N	Eikelboom type 021N	TCCCTCTCCCAAATTCTA	Wagner, M., Assmus, B., Hartmann, A., Hutzler, P. and Amann, R. 1994. In situ analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-targeted oligonucleotide probes and confocal scanning laser microscopy. Journal of Microscopy (Oxford) 176(3), 181-187.
G123T + Comp.G123T	*Thiothrix sp.*, Eikelboom type 021N groups	CCTTCCGATCTCTATGCA	Kanagawa, T., Kamagata, Y., Aruga, S., Kohno, T., Horn, M. and Wagner, M. (2000) Phylogenetic Analysis of and Oligonucleotide Probe Development for Eikelboom Type 021N Filamentous Bacteria Isolated from Bulking Activated Sludge. Appl. Envir. Microbiol. 66(11), 5043-5052
MNP1	nocardioform *Actinomycetes*	TTAGACCCAGTTTCCCAGGCT	Schuppler M., Wagner M., Schön G. and Göbel U. (1998). In situ identification of nocardioform actinomycetes in activated sludge using fluorescent rRNA-targeted oligonucleotide probes. Microbiology 144: 249-259

NLIMI 91	*Nostocoida limicola* I, *Streptococcus* spp.	CGCCACTATCTTCTCAGT	Liu J. R. and Seviour R. J. 2001. Design and application of oligonucleotide probes for fluorescent in situ identification of the filamentous bacterial morphotype Nostocoida limicola in activated sludge. Environ. Microbiol. 3: 551-560.
NLII65	*Nostocoida limicola* II, Actinomycetes	CAAGCTCCTCGTCACCGTT	Bradford, D. (1997). Molecular biological studies of filamentous bacteria associated with activated sludge bulking and foaming. Dissertation, Department Microbiology, University Queensland, Australia.
NLIMII 175	*Nostocoida limicola* II strains (except strain Ben 70)	GGCTCCGTCTCGTATCCG	Liu J. R. and Seviour R. J. 2001. Design and application of oligonucleotide probes for fluorescent in situ identification of the filamentous bacterial morphotype Nostocoida limicola in activated sludge. Environ. Microbiol. 3: 551-560.
Noli-644	Candidatus *Alysiosphaera europaea*	TCCGGTCTCCAGCCACA	Snaidr, J., Beimfohr, C., Levantesi, C., Rossetti, S., van der Waarde, J., Geurkink, B., Eikelboom, D., Lemaitre, M., Tandoi V. 2002. Phylogenetic analysis and in situ identification of "Nostocoida limicola"-like filamentous bacteria in activated sludge from industrial wastewater treatment plants. Water Sci Technol. 46:99-104. Levantesi, C., Beimfohr, C., Geurkink, B., Rosetti, S., Thelen, K., Kroonemann, J., Snaidr, J., van der Waarde, J., Tandoi, V. 2004. Filamentous Alphaproteobacteria associated with bulking in industrial waste wtaer treatmnet plants. Syst. Appl. Microbial. 27:716-727.
PPx3-1428	Candidatus *Alysiomicrobium bavaricum*	TGGCCCACCGGCTTCGGG	Snaidr, J., Beimfohr, C., Levantesi, C., Rossetti, S., van der Waarde, J., Geurkink, B., Eikelboom, D., Lemaitre, M., Tandoi V. 2002. Phylogenetic analysis and in situ identification of "Nostocoida limicola"-like filamentous bacteria in activated sludge from industrial wastewater treatment plants. Water Sci Technol. 46:99-104. Levantesi, C., Beimfohr, C., Geurkink, B., Rosetti, S., Thelen, K., Kroonemann, J., Snaidr, J., van der Waarde, J., Tandoi, V. 2004. Filamentous Alphaproteobacteria associated with bulking in industrial waste wtaer treatmnet plants. Syst. Appl. Microbial. 27:716-727.
MC2-649	Candidatus *Monilibacter batavus*	CTCTCCCGGACTCGAGCC	Snaidr, J., Beimfohr, C., Levantesi, C., Rossetti, S., van der Waarde, J., Geurkink, B., Eikelboom, D., Lemaitre, M., Tandoi V. 2002. Phylogenetic analysis and in situ identification of "Nostocoida limicola"-like filamentous bacteria in activated sludge from industrial wastewater treatment plants. Water Sci Technol. 46:99-104. Levantesi, C., Beimfohr, C., Geurkink, B., Rosetti, S., Thelen, K., Kroonemann, J., Snaidr, J., van der Waarde, J., Tandoi, V. 2004. Filamentous Alphaproteobacteria associated with bulking in industrial waste wtaer treatmnet plants. Syst. Appl. Microbial. 27:716-727.
ntspa1431	*Nitrospira* II (genus)	TTGGCTTGGGCGACTTCA	Maixner F., Noguera D. R., Anneser B., Stoecker K., Wegl G., Wagner M. and Daims H. (2006). Nitrite concentration influences the population structure of Nitrospira-like bacteria. Environ. Microbiol. 8 (8): 1487-1495.

ntspa1151	*Nitrospira* I (genus)	TTCTCCTGGGCAGTCTCTCC	Maixner F., Noguera D. R., Anneser B., Stoecker K., Wegl G., Wagner M. and Daims H. (2006). Nitrite concentration influences the population structure of Nitrospira-like bacteria. Environ. Microbiol. 8 (8): 1487-1495.
ntspa662 +comp.	*Nitrospira* (genus)	GGAATTCCGCGCTCCTCT	Daims H., Nielsen J. L., Nielsen P. H., Schleifer K. H. and Wagner M. (2001). In situ characterization of Nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants. Appl. Environ. Microbiol. 67: 5273-5284
Cluster 6a192 +comp.	*Nitrosomonas oligotropha* lin.	CTTTCGATCCCCTACTTTCC	Adamczyk J., Hesselsoe M., Iversen N., Horn M., Lehner A., Nielsen P. H., Schloter M., Roslev P. and Wagner M. (2003) The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. Appl. Environ. Microbiol. 69, 6875-6887.
NIT3 +comp.	*Nitrobacter* spp.	CCTGTGCTCCATGCTCCG	Wagner M., Rath G., Koops H.P., Flood J. and Amann R. (1996). In situ analysis of nitrifying bacteria in sewage treatment plants. Wat Sci Techn 34: 237-244
NEU+comp.	halop./t. *Nitrosomonas*	CCCCTCTGCTGCACTCTA	Wagner M., Rath G., Amann R., Koops H.-P. and Schleifer K.-H. (1995). In situ identification of ammonia-oxidizing bacteria. Syst. Appl. Microbiol. 18: 251-264.
Nsv443	*Nitrospira* spp.	CCGTGACCGTTTCGTTCCG	Mobarry B. K., Wagner M., Urbain V., Rittmann B. E. and Stahl D. A. (1996). Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Appl. Environ. Microbiol. 62: 2156-2162.
Efi58	*Enterococcus* sp.	TGACTCCTCTTCAGACTT	Behr T., Koob C., Schedl M., Mehlen A., Meier H., Knopp D., Frahm E., Obst U., Schleifer K., Niessner R. and Ludwig W. (2000). A nested array of rRNA targeted probes for the detection and identification of enterococci by reverse hybridization. Syst. Appl. Microbiol. 23: 563-572.
Efe1473	*Enterococcus* sp.	TAACTCTACTCAAGACTCAT	by Ludwig W. and Santos L., 2007, personal communication

Appendix D



Microscopic Investigations on the Structure and Composition of Aerobic Activated Sludge Granules
Silvia WEBER, Gerhard WANNER, Andreas HOFMANN, Wolfgang LUDWIG and Johannes FRIED
Iso
Aerobic activated sludge granules are spherical clusters of dense microbial biofilms used to improve sludge settleability and water purification in wastewater treatment processes. This study illustrates the development, structure and microbial composition of such granules by means of light microscopy, confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). Special focus is set on the role of stalked ciliates (Subclass Peritrichia) as important structure elements of the granular biofilm.
SBR-Cycle
Structure and Development of Granules in Sequencing-Batch-Reactors
The GRANULE
Biofilm Community
Fungi
Bacteri
Protozoa
FISH
CONCLUSIONS of this study:
1. The development from the floc to the mature granule takes place in three phases; 2. Stalked ciliates are always and fungi sometimes involved in that process; 3. A mature granule generally comprises a dense core zone and a loose structured fringe zone; 4. Density, compactness, size and microbial composition of a granule depend on the used wastewater and the operational setup of the sequencing-batch-reactor; 5. SEM, light microscopy and CLSM (in combination with FISH) are suitable methods to explore the structure of granules and to describe simultanousely the role of ciliates, bacteria and fungi within that process.

A modified FISH Protocol to analyse microbial communities in Aerobic Activated Sludge Granules

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Aerobic activated sludge granules are dense, spherical biofilms consisting of protozoa, bacteria, extracellular polymeric substances, and sometimes fungi. These granules can dramatically improve purification efficiency and sludge settleability in wastewater treatment. Recent studies showed that mostly ciliates (subclass Peritrichia) provide the structural matrix of such microbial aggregates. In the scope of this study a FISH (fluorescent in situ hybridisation) protocol was developed to examine the microbial composition of granules. That protocol shall help to enlighten the associations of protozoa, bacteria and fungi during the development and establishment of granules as well as the pattern of distribution of microorganisms inside the granule.
Granules were sampled from sequencing batch reactors (SBR) supplied with different wastewater (ww) types: Malthouse, brewery and glucose-peptone-based artificial ww (artificial ww: Two reactors with different aeration flow rates of 4l/min and 6l/min).

Adapting FISH on granules:

Aerobic activated sludge granules are compact spheric biofilms, which can reach a diameter up to 8 mm. It is therefore not possible to hybridise whole granules:

- Oligonucleotide probes can not reach the core of a granule during hybridization
- Microscopy of the granule´s core not possible due to insufficient focussing ability

Solution

Adaption of the FISH technique on thin sliced granules

The scheme below illustrates the new optimized protocol for sample fixation and preparation and FISH on sliced granules. Granules from different wastewaters showed different cutting ability. Malthouse and artificial ww granules could be sliced without any problems. The SBR approach with brewery ww resulted in fluffy and slimy granule-like biofilms due to many filamentous bacteria. Those aggregates could mostly not be sliced since a huge amount of polysaccharides in the biofilm inhibits the freezing process and therefore the following cryosectioning step. Fig. 4-9 present first hybridisation results with sliced granules from malthouse, brewery and artificial ww samples. In all hybridisations specific probe signals could be detected in the fringe as well as in the core zone of granules. In future experiments this new protocol will be very helpful to investigate the specific distribution pattern of bacteria, ciliates and fungi inside of different granular biofilms.

GRANULE – FISH

GRANULE PREPARATION

- **Fixation** - Step1: PFA 2%; Bouin 50%, 4°C, 20h | **Fixation** - Step2: 2h shaking on ice
- **Washing** (2x): 10 min shaking in 1 x PBS | **Dehydration**: 10 min each: 50%/80%/100% EtOH
- **Embedding**: 1h in cryomedium | **Freeze**: - 196°C, 3 min | **Store**: -80°C
- **Cryosectioning**: 20-30 µm slices, pre-cooled, coated slides

SLICING + FISH

- **Heat fixation**: 46°C, 1h | **Immobilization**: 1% agarose layer
- **Lysozyme treatment**: Room temp., 20 min, 4 x 10^4 U Lysozyme
- **Washing**: Rinse with pure water | **Dehydration**: Repeat as described above
- **FISH**: rRNA-directed probes for Bacteria and Ciliates | **Signal detection**: Confocal laser scanning microscopy

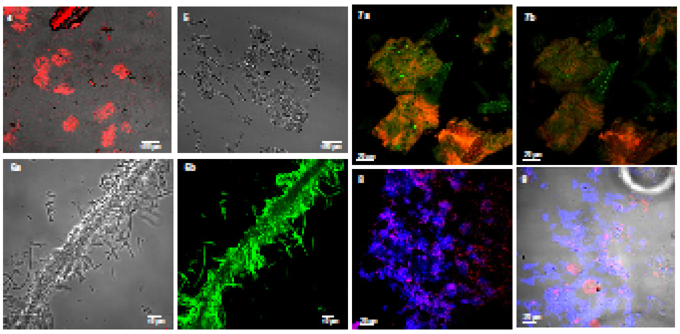

Fig. 4,5: (4) FISH on granule slice (30 µm, malthouse ww). Overlay of phase contrast and fluorescence image. Red Cy3-signals (eucaryotic specific probe EUK516) of truncated ciliate cells. (5) Negative control without probe. Overlay of phase contrast and fluorescence image. No signal or detectable autofluorescence of ciliate cells.
Fig. 6a,b: FISH on granule slice (30 µm, brewery ww). Phase contrast (6a) and fluorescence (6b) image. Green Fluos-signal (bacteria-specific probe EUB338-Mix): Bacteria colonizing a fungal hypha.
Fig. 7a,b: FISH on slices of a granule (30 µm, malthouse ww). Fluorescence images of two different sections of a stack (Δz: 0,42 µm optical slices). Green Fluos-signal (probe EUB-Mix): Bacteria on stalks and in the cells of ciliates. Red Cy3-signal (probe EUK516): Truncated ciliate cells.
Fig. 8: FISH on granule slice (25 µm, artificial ww, 4l/min). Fluorescence image. Blue signal: DAPI staining of complex bacterial biomass, red Cy3-signal of probe HGC69a, specific for Actinobacteria.
Fig. 9: FISH on granule slice (25 µm, artificial ww, 4l/min). Overlay of phase contrast and fluorescence image. Blue signal: DAPI staining of complex bacterial biomass, red Cy3-signal of probe Beta2a, specific for Betaproteobacteria.

FISH with homogenized granules

Since the cryosectioning procedure is rather time-consuming the overall bacterial composition of all granule types was determined first with homogenized samples. PFA 2% and 4% fixed biofilms were homogenized 10 sec. with the „Ultraturrax" and applied on gelatine coated slides. The samples were subjected to FISH with several 16S rRNA directed oligonucleotide probes specific for microorganisms which are supposed to occur in wastewater systems (for more information visit www.microbial-ecology.net/probebase). Probes and FISH results are listed in table 1. Fig. 10-12 show hybridisation examples.

Oligonucleotide probe 16S rRNA directed	Target Organism	Malthouse	Brewery	Artificial (4l)	Artificial (6l)
EUB338-Mix	most Bacteria	++	++	+++	+++
EUK516	most Eukarya	++	++	+++	+++
BET42a + Comp.	Betaproteobacteria	+	+	++	++
GAM42a + Comp.	Gammaproteobacteria	-	+++	++	-
Alf1b	Alphaproteobacteria, some Deltaproteobacteria, Spirochaetes	++	++	+++	+++
HGC69a	Actinobacteria	++	-	n.t.	+++
[illegible]	[illegible]	-	-	+++	+++
[illegible]	[illegible]	++	-	++	++
NSO190	[illegible]	n.t.	-	-	-
NSO1225	[illegible]	n.t.	-	-	-
Nmo218	[illegible]	n.t.	-	-	-
NmV	[illegible]	n.t.	-	-	-
[illegible]	[illegible]	n.t.	-	-	-
GNSB-941	phylum Chloroflexi (green nonsulfur bacteria)	n.t.	-	-	-
CFX1223	phylum Chloroflexi (green nonsulfur bacteria)	n.t.	-	-	-
[illegible] + Comp.	[illegible]	n.t.	-	-	-
[illegible]	[illegible]	n.t.	-	---	---
SNA + Comp.	Sphaerotilus natans	n.t.	+	--	-
CTE	Comamonas spp., Acidovorax spp., Hydrogenophaga spp., Aquaspirillum spp.	n.t.	-	n.t.	n.t.
ACA23a [illegible]	Acinetobacter sp.	n.t.	-	n.t.	-
TNI	Thiothrix nivea	n.t.	++	n.t.	n.t.
21N	Eikelboom type 021N [illegible]	n.t.	-	--	--
G123T + Comp.	Thiothrix eikelboomii, T. nivea, T. unzii, T. fructosivorans, T. defluvii, Eikelboom type 021N group I, II, III	n.t.	-	n.t.	n.t.
[illegible]	[illegible]	n.t.	-	n.t.	n.t.
[illegible]	[illegible]	+++	-	n.t.	-
[illegible]	[illegible]	n.t.	-	n.t.	n.t.
[illegible]	[illegible]	n.t.	-	n.t.	n.t.
[illegible]	[illegible]	n.t.	-	n.t.	n.t.

Tab. 1: Preliminary FISH results of all tested 16S rRNA directed oligonucleotide probes with granules from different wastewater reactors. Intensity of probe signal is indicated from --- (no signal) to +++ (very strong signal). N.t.: Probe not yet tested with this sample.

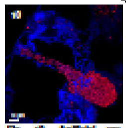

Fig. 10: Artificial ww (4l/min) Blue signal: DAPI staining of bacterial biomass, red Cy3-signal of probe Gam42a, specific for Gammaproteobacteria.

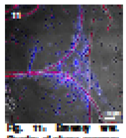

Fig. 11: Brewery ww. Overlay of phase contrast and fluorescence image. Blue signal: DAPI staining of bacterial biomass, red Cy3-signal of probe Beta2a, specific for Betaproteobacteria.

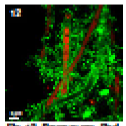

Fig. 12: Brewery ww. Red Cy3- signal (probe EUK516) of fungi, green Fluos-signal (EUB-Mix) of Bacteria.

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ISME 11, International Symposium on Microbial Ecology in Vienna, Austria

Composition and Architecture of Granular Wastewater Biofilms Affected by Ciliated Protozoa and Fungi

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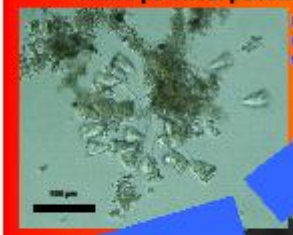
Ciliated protozoa are important consumers of bacteria and other microorganisms and often colonize and grow on substrates, thus forming biofilms, which can be found in almost every natural aqueous habitat. Microbial granules are spherical aggregates of microorganisms which nowadays are increasingly used in activated sludge processes to enhance sludge sedimentation and to reduce process time. It is presented on how ciliates affect granule architecture and total community composition, consisting mainly of ciliates, bacteria, and fungi. A new protocol for whole cell fluorescence in situ hybridisation was developed to differentiate taxonomic entities within microbial granules. This protocol combined with PCR-based techniques and with methods based on comparative morphology offers powerful possibilities to investigate and visualise the dynamics of complex microbial aggregates.



Colony of peritrichous ciliates, e.g. *Vorticella* sp., with attached bacteria

Freshly formed granule, overgrown by peritrichous ciliates

Observing Microbial Dynamics

Fixation: 2% PFA or 50% Bouin, 4°C, 20 h + 2 h shaking

Washing: 2 x 10 min shaking in 1xPBS

Dehydration: 50%, 80%, 100% EtOH, 10 min each

Embedment: Cryo-medium, 1 h

Freezing: -196°C, 3 min

Storage: -80°C

Cryo-Sectioning: 20-30 µm slices on pre-cooled, coated microscope slides

Heat-Fixation: 46°C, 1 h

Immobilisation: 1% agarose layer

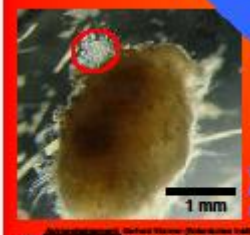
Lysozym-Treatment: Room-temp., 20 min, 4x10⁴ U Lysozyme

Washing: Rinse in pure water

Dehydration: As before

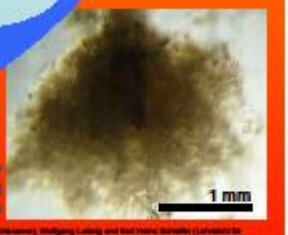
FISH: rRNA-directed probes; Fried et al., 2002

Identification: Epifluorescence, confocal laser scanning microscopy



Old granule densely filled with bacteria and overgrown with fungi and peritrichous ciliates

Mature granule, overgrown by peritrichous ciliates and densely filled with bacteria



SIL-Austria Treffen, 22.-24.10.2006, Innsbruck, Austria (SIL, Societas Internationalis Limnologiae)

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Curriculum Vitae

Name:	Silvia Weber
Address:	Behlertstr. 27A, 14469 Potsdam
Date of birth:	27.04.1978, München
Email:	silvi.weber@gmx.net

Professional experience:

July 2008 – present	Manager Business Development and Scientific Advisor, Aurigon Life Science GmbH Tutzing, Germany
June 2004 – present	PhD thesis, Technische Universität München, Department of Microbiology: „Molecular and microscopic studies on the diversity of protozoa, bacteria and fungi and their impact on the structural formation of aerobic sewage granules"
Aug. 1997 - Aug. 1998	Veterinary assistant, veterinary hospital Dres. Skorski & Wittmann, Neufahrn-Grüneck

Education:

May 2004	**German university diploma in Biology** Grade 1.7 (on a scale of 1 to 4, with 1 being the highest) "with distinction" *Major subject Microbiology, minor subjects Virology and Zoology*
April 2003 – May 2004 Munich	Diploma thesis at the Department of Microbiology, TUM, Grade 1.3 (on a scale of 1 to 4, with 1 being the highest) Advisor: Dr. Johannes Fried „Adaptation of fluorescence in situ hybridization techniques for the investigation of rumen ciliates" (EU sponsored project)
Nov. 1998 - April 2003	Biology Studies, TUM, Munich
Sept. 1988 - July 1997	A-levels (German Abitur), Dom-Gymnasium Freising, Germany

Languages:

German	native
English	business fluent
Greek	basics
Italian	basics

Memberships:

VBiol (Verband deutscher Biologen)
VAAM (Vereinigung für allgemeine und angewandte Mikrobiologie)
ASM (American Society for Microbiology)
TaBueKu (Verein für Tanz- und Bühnenkunst)
Sportschule/Akademie MiChi

Interests:

trainer Karate (1. Dan),

stage dance and acting

travel, languages, music

Munich, August, 2009

Silvia Weber (Dipl. Biol.)

Appendix to CV

Publications in peer-reviewed scientific journals:

Weber, S. D., Wanner, G., Ludwig, W., Schleifer K. H., and Fried J. (2007). Microbial composition and structure of aerobic granular sewage biofilms. *Appl. Environ. Microbiol.* 73: 6233-6240.

Weber, S. D., Hofmann, A., Pilofer, M., Wanner, G., Agerer, R., Ludwig, W., Schleifer K. H., and Fried J. (2009). The diversity of fungi in aerobic sewage granules assessed by 18S rRNA gene and ITS sequence analyses. *FEMS Microbiolgy Ecology 68(2): 246-254.*

Weber, S. D., Schwarzenbeck, N., Lemmer, H., Wanner, G., Ludwig, W., Schleifer K. H., and Fried J. (2009). Diversity, population dynamics and association of protozoa and bacteria in aerobic sludge granules from industrial wastewater sequencing batch reactors. *Submitted to FEMS Microbiology Ecology*

Conferences:

- VAAM (Vereinigung für Allgemeine und Angewandte Mikrobiologie) 2003, Braunschweig, Germany (poster presentation)
- DGP (Deutsche Gesellschaft für Protozoologie) 2004, Innsbruck, Austria und 2006, Berlin, Germany (oral and poster presentation)
- ISAM (International Symposium of Anaerobic Microbiology) 2005, Warsaw, Poland (oral and poster presentation)
- MEM (Munich Environmental Microbiology Meeting 2005, GSF, Munich, Germany (poster presentation)
- International Workshop – Development and control of functional biodiversity at micro- and macro-scales 2005, GSF Munich, Germany (poster presentation)
- ISME (International Symposium for Microbial Ecology) 2006, Vienna, Austria (poster presentation)
- ASM (American Society of Microbiology) - Conference on Biofilms 2007, Quebec, Canada (oral presentation as selected speaker)

Grants, invitations and prices:

Poster award (1. rank) at the DGP 2006, Berlin, Germany
Invitation for the talk at the ASM Conference on Biofilms 2007
Selected travel grant for the ASM Conference on Biofilms 2007

Personnel and project management:

2004-2008 within the scope of PhD thesis:

- project planning, coordination and training of a BTA
- planning, coordination and supervision of several bachelor theses and one diploma thesis
- supervision and mentoring of students (individuals and groups) and graduate assistants

2000 – 2001: Tutor for undergraduate students

1994 - 1995: Head of the student council, organization of intern school projects at the Dom-Gymnasium, Freising

Further trainings:

Seminars at the Carl-von-Linde-Akademie for scientific employees of the TU Munich:

- textcoaching
- leadership strategies for women
- basic course - presentation techniques

Internships accompanying the studies as graduate assistant 2000-2003:

- Institute of Soil Ecology, GSF- National Research Center for Environment and Health, Neuherberg/Munich
- Faculty of Life Sciences, Chair of Zoology, TU Munich
- Faculty of Life Sciences, Department of Microbiology, TU Munich (e.g. medical microbiology in the fields of *quorum sensing* und biofilm research)

Study trips including project work and seminars:

- flora-fauna-habitat of the Mediterranean area, Croatia, Rovinji, 2000
- flora, fauna and civilization of Sumatra, Indonesia, 2001