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Formation, Structure and Function of Aerobic Granular Sludge

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KURZFASSUNG

Im Vergleich zu belebtem Schlamm in Flockenform bietet granulierter Schlamm verschiedene Vorteile, vor allem besitzt er ausgezeichnete Absetzeigenschaften. Die Granulierung von anaerobem Schlamm ist gut dokumentiert und er wird erfolgreich in der anaeroben Abwasserreinigung eingesetzt. Derzeit werden Untersuchungen zum Potenzial von aerobem granuliertem Belebtschlamm durchgeführt. Erste Abwasserreinigungsanlagen im Labormaßstab sind bereits im Betrieb.

Ziel dieser Studie war die Ermittlung grundlegender Daten, um den Granulierungsprozess zu verstehen, zu kontrollieren und in seiner Effizienz zu steigern. Untersucht wurden Bildung, Struktur und Eigenschaften von aerobem granuliertem Belebtschlamm. Hierbei sollten ein Überblick über die mikrobielle Zusammensetzung, Informationen zur Abwasserreinigungs-kapazität, den Absetzeigenschaften und zu weiteren Charakteristika gewonnen werden. Zur Kultivierung aerober Granula sowie zur Untersuchung der notwendigen Prozessbedingungen wurden im Parallelversuch zwei Sequencing Batch Reaktoren (SBR) im Labormaßstab eingesetzt. Die SBR wurden mit unterschiedlichen synthetischen Abwässern betrieben. Die Prozessparameter, wie z.B. die Kohlenstoffquelle, die Absetzzeit oder die Volumenaustauschrate, wurden variiert.

Aerobe Granula wurden in den Labor-SBR hauptsächlich innerhalb von 15 bis 25 Tagen gebildet. Die Granulierung erfolgte sowohl unter aeroben als auch im periodischen Wechsel von anaeroben und aeroben Prozessbedingungen. Sucrose verhinderte als einzige der verwendeten bioverfügbaren Kohlenstoffquellen die Granulaentstehung. Mit Volumen-Austauschraten zwischen 50 und 70 %, kombiniert mit schnellem Befüllen und kurzen Absetzphasen konnte aerober granulierter Belebtschlamm in reproduzierbarer Weise hergestellt werden. Besondere Gründe für die Initialisierung der Granulierung konnten nicht identifiziert werden.

Um filamentöse Mikroorganismen nachzuweisen, wurden aerobe Granula regelmäßig bis zur zehnten Woche nach ihrer Entstehung lichtmikroskopisch untersucht. Die Ergebnisse zeigten, dass filamentöse Mikroorganismen im ersten Abschnitt des Granulierungsprozesses ein strukturelles Rückgrad der Granula bildeten. Dieses Rückgrad war jedoch im Laufe der weiteren Entwicklung für die Stabilität der wachsenden Granula offensichtlich nicht mehr notwendig.

Mit Hilfe von molekularbiologischen Methoden, insbesondere der Fluoreszenz-*In-Situ*-Hybridisierung (FISH) und dem „full cycle 16S rDNA approach“, war es möglich, die beteiligten filamentösen Mikroorganismen, *Sphaerotilus natans*, zu bestimmen. In stabilem Prozesszustand bestand die mikrobielle Population überwiegend aus Betaproteobakterien (ca. 89 %) und Gammaproteobakterien (ca. 5 %), die sich jeweils durch eine relativ geringe

Speziesvielfalt auszeichneten. Für die Bildung von Belebtschlammflocken typische Mikroorganismen (z.B. *Zoogloea ramigera*) konnten sich gut entwickeln. Die im stabilen Betriebszustand dominant vorkommenden *Zoogloea ramigera* dürften für die sehr kompakte Granulastruktur verantwortlich gewesen sein.

Um die biochemischen Prozesse in Granula zu charakterisieren, wurden Konzentrationsgradienten von Ammonium-, Nitrat- und Nitrit-Ionen sowie von Sauerstoff bestimmt. Unterschiedliche Mikrosensoren (Liquid-Ion-Exchanging-Membrane-Sensoren [LIX] und Mikrooptoden) sowie eine spezielle Durchflusszelle wurden eingesetzt, um einzelne Granula außerhalb des Reaktors zu untersuchen. Die Messungen ergaben, dass die Granulastruktur aus aktiven oberflächlichen Schichten bestand, die bis zu 500 µm tief waren. Mikroorganismen in tieferen Schichten waren deutlich weniger aktiv. Im inneren Kern konnte Diffusionslimitierung festgestellt werden. Zusätzlich dazu waren in tieferen Schichten anoxische und anaerobe Mikronischen sowie generell Kanäle und Höhlen zu erkennen.

Alle kultivierten granulierten Belebtschlämme setzten sich grundsätzlich schnell und vollständig ab. Die durchschnittliche Sinkgeschwindigkeit einzelner Granula betrug zwischen 10 und 40 m h^{-1} bei einem Schlammvolumenindex von 80 bis 20 ml g^{-1}. Im stabilen Betriebszustand betrugen die Granuladurchmesser zwischen 0,2 und 10,0 mm. Die Dichte von Belebtschlammflocken und aeroben Granula befand sich in einem ähnlichen Bereich. Das Absetzverhalten von Granula stand in direkter Beziehung zu ihrer Größe. Die Sinkgeschwindigkeit einzelner Granula konnte mit einer Variante des Stokeschen Gesetzes beschrieben werden. Im Vergleich zu Belebtschlamm in Flockenform verhakten sich Granula während des Absinkens nicht miteinander. Weder ein Verhaken von filamentösen Granulaoberflächen noch eine physikalisch-chemische Anziehung haben den Absetzprozess merklich beeinflusst.

Die Nährstoffreinigungsprozesse wurden durch einen effizienten CSB-Abbau sowie simultane Nitrifikation/Denitrifikation charakterisiert. Biologische Phosphoreliminierung konnte eindeutig nachgewiesen werden. Abwechselnde anaerobe/aerobe Prozessbedingungen führten zu hoher und stabiler EBPR- (Enhanced Biological Phosphorus Removal) Aktivität. Nitrifizierende Bakterien oder bekannte Phosphat akkumulierende Organismen (PAOs) konnten jedoch mittels molekularbiologischer Methoden nicht identifiziert werden. Der Feststoffgehalt lag zwischen 10 und 13 g SS l^{-1}.

Aerobe Granula wiesen, verglichen mit Belebtschlamm in Flockenform, eine ähnliche spezifische Oberfläche und vergleichbare Adsorptionseigenschaften auf. Gegenüber der Einwirkung von Scherkräften waren sie geringfügig weniger empfindlich. Bei Untersuchungen zur Sauerstoffaufnahme des granulierten Schlammes wurde eine hohe Aktivität festgestellt. Die Respirationsrate blieb im Batch-Test über einen Zeitraum von sieben Tagen auf hohem Niveau, obwohl die Belüftung auf ein Minimum reduziert wurde und keine anorganischen sowie organischen Nährstoffe zugeführt wurden.

Fasst man die Untersuchungsergebnisse zur Bildung und Struktur aerober Granula zusammen, lässt sich die Granulierung wie folgt beschreiben:

Die Bildung von aerobem granuliertem Belebtschlamm war durch zwei Hauptschritte charakterisiert. In einem ersten Selektionsprozess wurde Biomasse mit verbesserten Absetzeigenschaften im SBR aufkonzentriert. Dabei waren die Prozessbedingungen durch eine hohe Volumenaustauschrate, schnelles Befüllen und kurze Absetzzeit gekennzeichnet. In den sich bildenden Schlammaggregaten formten filamentöse Mikroorganismen ein strukturelles Rückgrad. In einer zweiten Phase wurden kleine kompakte Schlammaggregate – erste Granula – gebildet und wuchsen weiter. Vor dem Hintergrund zyklischer Prozessbedingungen resultierte das Gleichgewicht zwischen der mikrobiellen Wachstumsrate und Ablöseprozessen an der Granulaoberfläche in annähernd sphärisch geformten Granula. Im stabilen Betriebszustand war das filamentöse Rückgrad nicht mehr vorhanden und typische Flocken bildende Mikroorganismen dominierten die Biozönose.

ABSTRACT

Compared to flocculent activated sludge granular sludge offers several advantages, above all excellent settling properties. The granulation of anaerobic sludge is well documented and granular sludge processes have been successfully used for anaerobic wastewater treatment. The potential of aerobic granular sludge is presently investigated and first laboratory wastewater treatment systems exist.

Aim of this study was to collect basic data to understand, control and enhance the granulation process. Formation, structural aspects and function of aerobic granular sludge were studied. An overview of the microbial composition, information on the removal capacity, settling properties and additional further qualities of aerobic granules should be gained. The cultivation of aerobic granules and the necessary process conditions were investigated using two laboratory scale sequencing batch reactors (SBR). The SBR were operated in parallel using different kinds of readily biodegradable substrate. Process parameters, e.g. type of carbon source, settling time or exchange ratio, were varied.

Using SBR systems aerobic granules could mainly be cultivated within 15 and 25 days. Granulation was possible for aerobic and for alternating anaerobic/aerobic conditions. Although all used carbon sources can be defined as readily biodegradable, wastewater with sucrose prevented granula formation. Volumetric exchange ratios in the range of 50 to 70 % combined with fast fill and short settling proved to be useful to cultivate aerobic granular sludge in a reproducible way. Explicit reasons for the initiation of the granulation could not be identified.

To check for filamentous microorganisms aerobic granular sludge was regularly examined by light microscopic observations. The results clearly indicated that filamentous microorganisms build a structural backbone in the first step of the granulation process. Examinations which took place up to the tenth week after the granulation showed that the filamentous backbone of the initial granules vanished during the first weeks. The backbone then was obviously no longer necessary for the structural stability of granules which grew further.

Using molecularbiological methods, especially fluorescence-*in-situ*-hybridisation (FISH) and the full cycle 16S rDNA approach, it was possible to determine the structurally involved filamentous bacteria, *Sphaerotilus natans.* In steady state conditions most of the population consisted of betaproteobacteria (approx. 89 %) and gammaproteobacteria (approx. 5 %), both characterised by a relatively limited diversity of species. Typical floc forming bacteria (*Zooglea ramigera*) were able to grow and dominate the microbial structure without destabilising the granula structure. Most likely, *Zooglea ramigera,* which were the dominating species in steady state conditions, were responsible for a very compact granula structure.

In order to characterise the biochemical processes inside granules concentration gradients of ammonium-, nitrite- and nitrate-ions and of oxygen were measured with different

microsensors (liquid-ion-exchanger-sensor (LIX) and microoptodes). A special flow-cell was used to study single granules harvested from the reactor. Granular structure was characterised by active surface layers which had a depth of around 0-500µm. Microorganisms in deeper layers were clearly less active and probably in stationary growth or in decay phase. In the inner core diffusion limitation occurred. Additional anoxic and anaerobic microniches existed and channels and voids could be detected.

For all aerobic granular sludges which were cultivated using a variety of synthetic wastewater compositions, generally, a fast and complete settling was observed. The average settling velocity of single granules reached 10 to 40 m h^{-1} at a SVI between 80 and 20 ml g^{-1}. Granula diameter in steady state conditions ranged from 0.2 to 10 mm. Buoyant density of flocculent and granular activated sludge was in a similar range. The granules' settling was related to their aggregate size. The settling velocity of single granules could be described by modified Stokes' law. Compared to flocculent activated sludge, granules did not coagulate while settling. Neither an entanglement of the filamentous granula surface nor physico-chemical attraction did influence the sedimentation process significantly.

The nutrient removal capacity was characterised by efficient COD removal combined with simultaneous nitrification/denitrification. A biomass content up to 10-13 g SS l^{-1} could be realised. The potential for enhanced biological phosphorus removal could be shown. Alternating anaerobic/aerobic conditions induced high and stable EBPR activity. However, no nitrifying bacteria or known phosphate accumulating organisms (PAOs) were identified by molecularbiological methods.

In comparison to flocculent activated sludge, a similar specific surface area and related adsorption properties were determined in batch experiments whereas shear sensitivity was slightly lower. Oxygen uptake tests of the granular sludge indicated high activity. The respiration rate maintained a high level for seven days although aeration was limited to a minimum and organic and inorganic nutrient supply was stopped.

Summarising the formation and structure of aerobic granular sludge the granulation process can be described as follows:

Aerobic granular sludge formation was characterised by two main stages. As initial selection step biomass with improved settling properties was concentrated in the reactor using high volumetric exchange ratio combined with fast fill and short settling. Filamentous microorganisms formed a structural backbone inside the first sludge aggregates. Secondly, the small and compact activated sludge aggregates - first granules - were initiated and grew further. The cyclic process and a balance between growth rate and detachment processes resulted in rather spherical granules. In steady state conditions the filamentous backbone vanished and typical floc-forming bacteria were dominant in the granular structure.

1 INTRODUCTION

The efficiency of activated sludge treatment systems is mainly determined by two factors, first of all the metabolic capability of the growing microorganisms and secondly the settling properties of the activated sludge aggregates, usually flocs. Sludge separation takes place in a clarifier, which can be a critical step in the activated sludge treatment process. To achieve reasonably good settling of the biomass sludge flocs have to coagulate to large and compact floc aggregates in the clarifier by means of adhesion forces. Basically, there are two main types of settling problems: (1) bulking sludge due to the excess growth of filamentous organisms and (2) poor flocculation or floc formation properties of the microorganisms. Foaming and scumming are other difficulties that are related with the massive growth of certain bacteria. Pin-point flocs may develop, which do not settle well by gravity.

Generally flocs settle the faster the larger and the more dense they are. Activated sludge flocs grow typically in a range of 30 to 1800 μm in diameter (Hilligardt and Hoffmann, 1997) but have a density that is only slightly higher than water density. Additionally flocs and floc aggregates hinder themselves while settling and therefore the overall sedimentation velocity is rather low. The result of poor separation properties is often loss of suspended solids into the effluent. If the sludge compacts poorly, recycling of the solids is hampered, thereby the treatment efficiency is reduced. In case of deteriorating sludge settling properties hydraulic loading of the system has to be lowered or the settling time has to be prolonged, if flow conditions in the tank can not be further improved. In practice, there are often high expenses to build and operate effective settling tanks, to avoid discharge of organic matter into the effluent and violation of COD discharge limits.

Alternatives for classical activated sludge systems could be based on compact reactors combined with an improved sludge that settles fast. Different attempts have been made to cultivate a compact form of activated sludge, granular activated sludge, in aerobic treatment system. Anaerobic granular sludge is known to exhibit good settling properties, good solid-liquid separation, high biomass retention, high activity, and an ability to withstand high loading rates. Hence several working groups tried to grow aerobic granular activated sludge according to the principle of anaerobic granular sludge.

Generally activated sludge systems are well investigated and data about particle size, wet density and settling properties of activated sludge flocs are available (e.g. Dammel and Schroeder, 1991; Echeverría *et al.*, 1992; Das *et al.*, 1993; Barbusinski and Koscielniak, 1995; Hilligardt and Hoffmann, 1997). However, to develop an alternative wastewater treatment system based on aerobic granular sludge basic data about the formation and structure of granular aggregates is necessary. Further, information to enhance and control the granulation process would be beneficial. Sludge structure and solid-liquid separation can be affected by changes in the operation of the treatment plant (e.g. Glasgow and Liu, 1991;

Barbusinski and Koscielniak, 1995; Knoop and Kunst, 1998). For instance, high hydrodynamic forces might limit the floc size, which is one determining factor for settling properties of the sludge.

The parameters influencing the floc formation of activated sludge are only known to some extent, their relative significance is still largely unknown. The floc formation of activated sludge is an active process and depends on physical, chemical, and biological factors. Complexity of the floc formation process is related to various parameters that interact (e.g. Ericsson and Eriksson, 1988; Gregory, 1989; Mozes *et al.*, 1989; Eriksson and Alm, 1991; Jorand *et al.*, 1994; Jorand *et al.*, 1995; Higgins and Novak; 1997c).

Granulation of anaerobic sludge has been well documented, especially in upflow anaerobic sludge blanket (UASB) reactor (e.g. Lettinga *et al.*, 1980; Dolfing *et al.*, 1987; Kosaric and Blaszczyk, 1990). Hence the study of aerobic granulation might profit from the existing data and experience regarding anaerobic granular sludge. For anaerobic granules Wirtz and Dague (1996) stated that a short hydraulic retention time (HRT) and a relatively high organic loading rate (OLR) are beneficial to granulation. It is generally thought that the upflow velocity in an UASB reactor creates a selective pressure to which the organisms have two responses: (1) being washed out or (2) being bound together and form easily settleable granules. There is indication that certain microorganisms within an aggregate may be selected by the hydraulics of the system as a possible protection mechanism (Guiot *et al.* 1992) and granular sludge may result from a change in the microbial population (Van der Hoek, 1987).

Regarding aerobic wastewater treatment systems less researchers investigated granulation. Aerobic granules could be successfully cultivated in a sequencing batch reactor (e.g. Morgenroth *et al.*, 1997; Beun *et al.*, 1999; Etterer and Wilderer, 2001). Beun (2001) observed spontaneous development of aerobic granules in an airlift suspension reactor. These first studies were promising and encouraged further investigations. Having in mind the crucial settling process in activated sludge systems aerobic granular sludge might become an interesting alternative to activated sludge flocs.

This study of aerobic granular sludge was organised as follows:

- Review of literature with focus on microbial aggregates in wastewater treatment systems. Data about the formation, structure, physico-chemical properties and the possibilities to investigate activated sludge flocs and microbial aggregates in general were collected.
- Assessment of sequencing batch reactor (SBR) technology to cultivate aerobic granules; operation of laboratory scale SBR using different readily degradable substrates; system optimisation by varying important process parameters (type of carbon source, settling time, organic loading, exchange ratio, total cycle time).
- Assessment of the time required to set granular sludge dominating in the reactor after system start-up; study of the effects of different process conditions on the granulation process.
- Evaluation of transport processes within granula using microelectrode measurements to determine gradients of dissolved oxygen, nitrate, and ammonium.
- Investigation of the response to variations in operation: (1) response to fully aerobic operation favouring the growth of heterotrophic bacteria, (2) increase of the carbon load, (3) response to partly anaerobic/aerobic operation for enhanced nutrient removal by favouring growth of phosphate accumulating organisms (PAO) and denitrifying bacteria.
- Investigation of physico-chemical parameters that influence settling characteristics and mass transfer.
- Investigation of the microbial population and their spatial distribution in granula using fluorescence-in-situ-hybridisation (FISH) and confocal laser scanning microscopy (CLSM).

2 BACKGROUND

2.1 Wastewater Treatment

In biological wastewater engineering, treatment systems were developed that select for the most suitable microbial community, and achieve the desired treatment both reliably and economically. The microbial consortium selected must have a proper metabolic activity which allows to meet the effluent quality requirements while producing a suitable biomass structure (e.g., floc, granule) that allows easy separation from the treated effluent (Wilderer *et al.*, 2001).

Wastewater can vary widely in flow, composition and concentration due mainly to the variations of the municipal activities in the catchment area of the treatment plant, and due to actual weather conditions. Wastewater contains organic and inorganic components in a complex mixture of compounds, both dissolved and solid. In the treatment plant, the contaminants must be eliminated to acceptably low concentrations. The basic principle of a wastewater treatment plant (WWTP) is to convert dissolved compounds into solids that can be effectively separated from the water phase. The type of conversion process chosen depends mainly on the kind and amount of wastewater to be treated as well as on economic and environmental considerations. For domestic wastewater, a combination of mechanical, biological and chemical treatment is normally used. Wastewater is generally treated in a series of steps or unit processes (Tchobanoglous and Burton, 1991).

2.1.1 The Activated Sludge Process

The most widespread biological wastewater treatment method is the activated sludge process which has been developed in England by Arden and Lockett in 1914. This process has been subjected to many improvements throughout the years. Basically, the classical activated sludge process consists of an aerated suspension of a mixed bacterial culture which carries out the metabolic conversion of the contaminants, but anoxic and anaerobic phases are common as well. The mixed bacterial culture is supposed to form flocs. A crucial point of this process is an effective solid-liquid separation. Sludge production is influenced by the type of process used, the operation of the plant and the wastewater characteristics. The recycling of sludge ensures a continuous inoculation and extends the sludge residence time. (Henze *et al.*, 1997; Wilen, 1999).

The efficiency of the activated sludge process is strongly linked to the ability of the sludge aggregates to settle. The produced effluent should be clear and the excess sludge should be easy to dewater. The activated sludge process can be realized in different ways. Biological nitrogen and phosphorus removal can be achieved by expanding a classical activated sludge

treatment plant with anoxic and anaerobic tanks in various configurations. Nitrogen removal is achieved by means of a two step process: nitrification and denitrification. Nitrification requires aerobic conditions. In contrast denitrification takes place under anoxic conditions with the final production of nitrogen gas. Biological phosphorus removal is carried out by phosphorous accumulating bacteria which can take up additional phosphate and store it internally. The enhanced biological phosphorus process (EBPR) needs a regular change of anaerobic and aerobic conditions (Henze *et al.*, 1997). The collective metabolic activities of the microbial community are:

- Conversion of soluble and colloidal organic materials into cell mass, carbon dioxide, water, and soluble microbial by-products, mostly monitored as COD removal.
- Oxidation of ammonia-nitrogen into nitrite-nitrogen and further into nitrate-nitrogen, commonly referred to as nitrification.
- Reduction of nitrate-nitrogen into nitrite-nitrogen and further into nitrogen gas, commonly referred to as denitrification.
- Accumulation of phosphorus beyond that needed for normal cell growth for disposal with excess biomass (i.e., the waste activated sludge), commonly referred to as enhanced biological phosphorus removal (EBPR) or simply „Bio-P removal“.

2.1.2 SBR Concept and Process Description

One variation of the activated sludge process is the sequencing batch reactor technology (Morgenroth and Wilderer, 1998).

The term SBR is used as a synonym for variable volume, periodic process, suspended growth, biological wastewater treatment technology. The most important differences compared to continuous flow activated sludge systems are (Wilderer *et al.*, 1997; Wilderer *et al.*, 2001):

- The influent and effluent streams are uncoupled.
- Biomass separation occurs in the biological reactor and not in a separate clarifier.
- The unit operations and unit processes that take place in each reactor follow each other in a time sequence that is progressively repeated in a `periodic` manner and not from tank to tank as they do in space-oriented systems.
- A portion of the treated water is periodically discharged from each tank to make room for a new batch of wastewater.

The SBR process is characterised by a series of process phases (e.g., fill, react, settle, decant, and idle) each lasting for a defined period of time. Sludge wasting normally takes place after settle, weekly, daily or during each cycle. The different phases of SBR operation are represented in Figure 1.

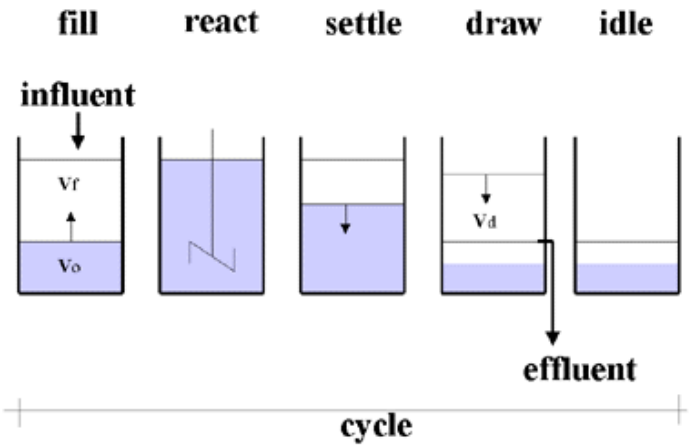


Figure 1. Operation phases following each other during one cycle of the generic SBR process (adapted from Wilderer *et al.*, 2001).

Thus, a SBR process is basically characterised by the duration of the different phases, total cycle time, fill time ratio (FTR), volumetric exchange ratio (VER) and hydraulic residence time (HRT). In addition, process parameters apply that are typical for activated sludge or biofilm systems. For instance, design and operation of an activated sludge SBR includes considerations of key factors such as sludge age, and sludge loading.

Depending on the mode of operation, an SBR system can be compared with a plug-flow reactor (PFR) or a completely mixed flow reactor (CMFR, see Weber and DiGiano, 1995). The mathematical representation of the SBR with fast fill is the same as that for the plug flow reactor at steady state, where the hydraulic residence in the PFR compares to `clock´ time in a completely mixed batch reactor (Wilderer *et al.*, 2001).

In discontinuously fed systems, e.g. sequencing batch reactor systems, microorganisms experience phases with external substrate availability (feast period) and phases without external substrate availability (famine period) (Beun, 2001). Van Loosdrecht *et al.* (1997) stated that microorganisms in general respond to feast-famine regimes by accumulating storage polymers when substrate is present. The storage polymers, usually glycogen, lipids or polyhydroxyalakanoates (PHA), are used for growth when the external substrate is depleted. In this way the organisms are capable of balancing their growth.

In continuously fed complete mixed tanks all substrate is converted to biomass at a constant rate and no storage polymers are formed. Studying the anoxic poly-ß-hydroxybutyrate (PHB) metabolism in activated sludge cultures of a SBR, Beun (2001) concluded that a large amount (70 % for synthetic wastewater) of the readily degradable substrate is used for synthesis of PHB. Using a fast filling strategy the organisms take up the substrate at a high rate, the specific growth rate increases and growth on the stored PHB is observed. Growth rate on PHB is clearly lower then on the original substrate (Majone *et al.*, 1999; Beun, 2001). This results in a balanced metabolism when sudden changes in substrate addition occurs.

If the substrate concentration suddenly rises, after previously growing under limited substrate conditions, microorganisms are able to rapidly take up substate. The substrate can not directly be converted by the growth processes in the cell, but the organisms can adjust their growth rate.

In pulse-wise fed systems, like in SBR, storage processes can play a dominant role. If microorganisms observe regularly periods with low or no substrate, bacteria capable of balancing their growth independent of the external substrate concentration might be enriched. This could be a competitive advantage, since bacteria not capable of substrate storage will have to invest extra energy for rapid growth in feast conditions. In famine conditions they will encounter problems in maintaining all their cell functions and in maintaining their structure in proper shape (Van Loosdrecht and Henze, 1999). The selected bacteria will have a competitive advantage and keep all cell systems viable even when the external substrate is consumed.

2.1.3 Periodic Processes

The strategy to control filamentous organisms and to promote the growth of a compact, good settling biomass are recognised as critical performance factors in activated sludge systems. Chudoba *et al.* (1973) showed that cyclic change of substrate concentrations is a selection factor in favouring certain microorganisms or repressing others, which can be strains of filamentous bacteria. They could demonstrate that filamentous bulking can sometimes be avoided by exposing activated sludge organisms periodically to high and low substrate concentrations. Chiesa and co-workers (Chiesa and Irvine, 1985; Chiesa *et al.*, 1985) and Wilderer and Schroeder (1986) confirmed these results.

In summary it is important to recognise (Wilderer *et al.*, 1997; Wilderer *et al.*, 2001):

- how selective pressures can be enforced and how they relate to growth conditions and growth rate differences that minimise or maximise the relative abundance of certain species or strains of microorganisms.
- that the relative formation of biological storage products relates to initial reaction conditions S_o/X_o (the highest ratio of substrate to microorganisms) and the previous history of the biomass.
- that most filamentous bacteria that have been isolated have both a slow or negligible capacity to use nitrate-nitrogen as an electron acceptor and a low soluble substrate storage capacity.
- that some filamentous bacteria have relatively high growth rates at elevated substrate concentrations and relatively high decay rates in the absence of substrate causing them to be at a competitive disadvantage relative to floc-formers when feast/famine conditions are imposed.

2.2 Activated Sludge

2.2.1 Biological Processes

There are very different kind of organisms responsible for the processes related to wastewater treatment. The situation at each treatment plant is different and the microbial community composition is directly related to the external conditions. In principle the organisms in biological treatment plants can be divided into Bacteria, Archea, fungi, algae, protozoa and metazoa. In activated sludge plants bacteria are dominant and algae as well as fungi play a minor role. The higher organisms of the group of protozoa graze on the bacteria and fungi. They occur in varying numbers depending on the nutrient situation of the plant. The particular role of protozoa regarding the overall nutrient removal is not intensively studied, but by taking up particles protozoa surely contribute to get clear effluents. Metazoa, e.g. *Rotifiers*, correspond to the total amount of protozoa (Henze *et al.*, 1997). The population of microorganisms present in the activated sludge depends on factors such as the composition of the wastewater, microbial growth rate, electron acceptor (oxygen or nitrate), pH or temperature. Settling, flocculation and floc formation characteristics also determine the microbial diversity, since microorganisms with slow settling can be washed out from the system. Bacteria can grow in three basic modes: dispersed, floc-forming and filamentous. In general the activated sludge process is very adaptive to changing compositions of the influent, a characteristic that makes the process highly efficient.

2.2.2 Composition of Flocs

Activated sludge aggregates are heterogeneously structured. The size of activated sludge flocs can range from a few microns up to 2000 µm (Li and Ganczarczyk, 1991; Hilligardt and Hoffmann, 1997). Bacteria are mainly responsible for the degradation of organic material in wastewater. Referring to biomass content and activity, bacteria are predominant. The microorganisms and inorganic particles are embedded in a matrix of extracellular polymeric substances (EPS) (Whitfield, 1988; Wingender *et al.*, 1999, Flemming and Wingender, 2000). This extensive network of polymers is cross-linked by physical, chemical or mechanical means (Eriksson *et al.*, 1992; Li and Ganczarczyk, 1993; Higgins and Novak, 1997c; Snidaro *et al.*, 1997; Zhang *et al.*, 1998). Hence different types of bacteria, dead cells, particulate organic and inorganic material and EPS make up the constituents of sludge flocs and granules.

Different authors (Frølund *et al.*, 1996; Nielsen *et al.*, 1996; Huber *et al.*, 1999; 2000) reported that the chemical composition of the organic matter of the sludge flocs changes with wastewater composition and treatment plant operation. Proteins and polysaccharides are main components of the organic substances in activated sludge.

2.2.2.1 Origin and Definition of Extracellular Polymeric Substances

EPS proteins and polysaccharides can be of various origin, e.g. compensation of inner- to extracellular concentration differences (Frolund *et al.*, 1994), products of bacterial lysis or storage polymers, caused by variations in the process (Van Loosdrecht and Heijnen, 1997; Majone *et al.*, 1999). Substances released during endogenous respiration at the beginning of starvation processes or substances produced during cell growth (Wingender *et al.*, 1999) can be EPS. The biopolymers can be adsorbed from the water phase, actively secreted, detached from the outer cell membrane or they are lysis products.

The production of EPS is a general property of microorganisms in natural environments and has been shown to occur both in prokaryotic (Bacteria, Archaea) and in eukaryotic (algae, fungi) microorganisms (Costerton *et al*, 1987; 1995; Wingender *et al.*, 1999). In EPS compounds of the wastewater and metabolic products of the microorganisms can be found. Organic compounds can be adsorbed and additional to the regular metabolism lysis of bacteria is a possible source of proteins, polysaccharides and lipids which can be a part of the extracellular matrix. EPS were also defined as "organic polymers of microbial origin which inactivated sludge flocs and biofilm systems are frequently responsible for binding cells and other particulate materials together (cohesion) and to the substratum (adhesion)" (Characklis and Wilderer, 1989). There can be two forms distinguished, capsular polymers and slime. The capsule can be found close to the cell surface, while the slime layer is more loosely attached to the cell.

2.2.2.2 Function and Transport of EPS

EPS have many different functions, such as retaining exoenzymes near the cell surface (Frolund *et al.*, 1995) and the binding of organic matter; they are also important for the attachment of cells to surfaces (Fowler, 1988). Different authors documented the synthesis and active transport of biopolymers by bacteria, a brief summary is given by Wingender *et al.* (1999). Different transport mechanisms have been identified, which indicates the potential for active bacterial regulation of the EPS production. EPS can be modified or degraded biotically or abiotically. Different enzymes, which are involved in the degradation of polymers, have been identified in biofilms and they are a common part of the EPS proteins. Therefore bacteria have on the one hand the possibility to produce biopolymers inside the cell and transport them outside the cell, but on the other hand these extracellular polymers can be modified and regulated by extracellular enzymes. Additional inorganic particles can be embedded, thus the mass increases and settling properties are improved.

It can be summarised that proteins are the dominating compounds of EPS (Zhang *et al.*, 1998; Huber *et al.*, 2000). The chemical composition and structure of EPS varies significantly, because the polymers are produced by different organisms under varying nutrient and

hydrodynamic conditions (Uhlinger and White, 1983; Huber *et al.*, 1999; 2000; Wingender *et al.*, 1999).

2.2.3 Floc Structure

In a first aggregation step microflocs composed of solid particles are built. Secondly those microflocs are the base for larger macroflocs with a three-dimensional network directly linked with a change in porosity. Flocs are therefore not homogenous, but consist of many smaller and larger units, particles and bacterial colonies (Snidaro *et al.*, 1997). The size of the aggregates is influenced by shearing effects caused by hydrodynamic forces in the mixed suspension. The larger the total attraction forces between particles, bacterial colonies and extracellular polymeric substances are the better is the chance for the aggregate to grow.

Physical stress occurs through hydrodynamic forces. Detachment processes balance the growth of biomass in steady-state and can be defined as the transport of particles from the sludge aggregate to the fluid phase. Different detachment processes can be noted, mainly erosion and floc break-up. Erosion is characterised by removal of small groups of cells from the surface, caused by shear forces of the moving fluid.

Based on literature review Wilén (1999) suggested a schematic description of an activated sludge floc (Figure 2).

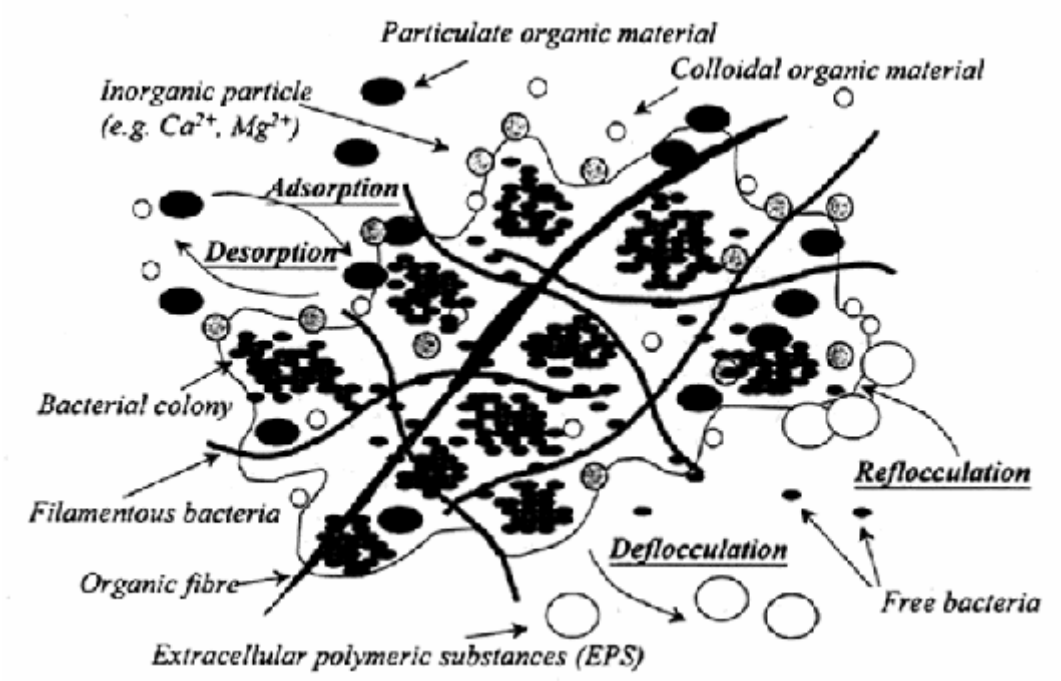


Figure 2. Schematic drawing of an activated sludge floc (Wilén, 1999).

Influent composition and other environmental factors may influence the chemical and microbial composition, hence the floc structure can be affected. In previous studies (Li and

Ganczarczyk, 1990; Nielsen and Keiding, 1998; Wilén, 1999) indications were collected supporting this thesis. Sulphide causes certain filamentous sulphide-oxidising bacteria to grow which leads finally to a Fe(III)-reduction (Nielsen *et al.*, 1997). This leads to a deflocculation of activated sludge because of the lower valence and higher solubility of Fe(II) (Nielsen and Keiding, 1998). Barbusinski and Koscielniak (1995) showed that the substrate loading affects the floc structure. With higher loading an increase of the average floc size could be determined. Further the growth of filamentous bacteria was enhanced leading finally to bulking sludge. Structural parameters were previously determined by a light scattering approach (Guan *et al.,* 1998). This technique, though fast, provides only an indirect insight on physical floc properties. A direct, but time consuming, approach, on 3D-modeling of activated sludge flocs was published by Zartarian *et al.* (1997). Images of a cryo-sectioned sludge floc were taken and a 3D reconstruction applied.

Sludge flocs might be defined as suspended biofilm. Biofilm studies indicated that surface loading and shear effects should be considered regarding structural aspects (Van Loosdrecht *et al.*, 1995). This effects might be similar regarding sludge flocs. The ratio between surface loading and shear rate is suggested to be the essential environmental factor determining the steady state biofilm structure. Additionally, characteristics of the individual organisms, such as yield and growth rate, play an important role. Parallels to sludge aggregates exist and in principle growth conditions at the aggregate surface should be similar, hence floc formation and granulation research could directly profit from biofilm related modeling. Although originally designed for biofilm research 3-D models may help to understand floc formation and structure. Picioreanu *et al.* (1998a; b) and Noguera *et al.* (1999) described biofilm growth using cellular automation models. Furthermore, biofilm growth on a spherical carrier under mass transfer limiting conditions was simulated by Picioreanu (1998a; b).

High floc strength can be explained by increased adhesion due to additional connections between the particles compared to very porous floc structures. Furthermore, the EPS composition is decisive for the floc structure and strength. If electrostatic forces are dominant floc strength is lowest. Strong aggregates are glued together by high molecular substances. Thus, the total of different forces between the polymer chains and the primary particles is crucial (see also section 2.2.4).

2.2.4 Floc Strength

The aggregate strength is crucially determined by its origin. Flocs, developed under high hydrodynamic stress conditions have according to Mihopulos (1995) a lower porosity and better ability to stand physical treatment, e.g. pumping. The term floc strength has frequently been used and suggests that flocs of low strength are relatively vulnerable to produce colloids[1]

[1] Colloids are small particles suspended and dispersed through a different medium. The physico-chemical properties of colloids can not adequately be described as solid state. Typical for colloidal properties is a particle diameter range of 10^{-7} to 10^{-9} m (Deryagin and Landau, 1941; Schramm, 2001).

when exposed to turbulence, but no firm characterisation method exists with respect to the release of colloids. The main fraction of sludge particles by mass or volume is contributed by sludge flocs around 25-200 μm in size (Hilligardt and Hoffmann, 1997). However, a fraction of the sludge particles are sized around 0.5-5 μm (Galil *et al.*, 1991; Parker *et al.* 1970, 1971; Li and Ganczarczyk, 1990, Snidaro *et al.*, 1997; Jorand *et al.*, 1995). Due to their small diameter, they have colloidal properties (Schramm, 2001), i.e. they do not settle under gravitation forces.

The particles sized around 0.5-5 μm represent the smallest units or building blocks of the sludge suspension, and may be termed singlets, primary particles or sludge colloids. The strength of particle interactions in flocs – usually termed floc strength – has been characterised in a number of ways by means of either floc fragmentation or erosion processes. Sustainable floc size or the level of turbulence required for fragmentation has been used as indicators of interaction energy (Glasgow and Hsu, 1982; Leentvar and Rebhun, 1983; Bache and Al-Ani, 1989; Glasgow and Liu, 1991). Interpretation of such data is, however, complicated by the influence of fractal dimension on the floc size (Gregory, 1989). A problem of such methods is that the phenomenon of fragmentation is mainly hydrodynamic and has little to do with physico-chemical phenomena. These methods are mainly relevant for processes with a critical floc size (Mikkelsen, 1999).

Based on the semi-theoretical model of simultaneous flocculation and erosion developed by Argaman and Kaufman (1968) and extended by Parker *et al.* (1970) Wahlberg *et al.* (1994) and Mikkelsen *et al.* (1996) investigated floc formation and floc strength. According to several studies of Mikkelsen and Keiding (1999; 2000 submitted) and Mikkelsen (1999; 2001) it is useful to study supernatant turbidity or sludge filterability in response to turbulence or sonication energy. Such methods include the measurement of the slope of capillary suction time (CST) versus shear time (Spinosa and Mininni, 1984; Eriksson *et al.*, 1992) or the slope of turbidity versus shear time (Nielsen and Keiding, 1998). The slope and sonication methods are purely empirical. Mikkelsen and Keiding (1999) investigated the possibility for characterisation of sludge floc strength with a physico-chemical basis. They developed a model for the release of colloids from sludge flocs exposed to turbulent shear which predicts the equilibrium colloid concentration in response to sludge solid content, turbulent shear and the interaction energy (or binding strength) between the sludge colloids and flocs. The model is briefly explained in the following.

In activated sludge systems normally some extent of dispersion is found, as the particle size distribution is bimodal, containing both primary particles and flocs of sizes approx. 0.5-5 μm and 20-200 μm, respectively (Parker *et al.*, 1970). For colloid concentrations well above the critical concentrations of flocculation we may expect equilibrium to be established by the balance of continuous adsorption and desorption, governed by the interaction energy (ΔH) of the adsorption process. This can be also expected for activated sludge, but the interaction

energy can not be estimated by addition of the implicated surface forces, because of the high system complexity. Assuming that the nuclei of adsorption are no larger than the largest of the sludge components, a sludge floc can be regarded as a multi-layer adsorption phenomenon. For this system there is no reason to expect two adsorption energies belonging to the first and following adsorption layers. With this applying to all adsorption layers, however, an approximation assuming only one (average) interaction layers as in the Langmuir isotherm may be reasonable. Although mathematical identical to the Langmuir isotherm, the described model is conceptually different, i.e. the adsorption-desorption equilibrium is governed by the existence of one (average) energy of adsorption and a critical adsorption level exists.

Hence, shear tests offer a possibility to draw comparisons between the physico-chemical understanding of the equilibrium and the equilibrium observed in practice. In a defined shear test the difference in physical properties of the singlets and flocs may be utilised to separate them, as flocs settle in a gravitational field, while singlets remain dispersed in solution. Thus, the evolution of supernatant turbidity of dispersed mass concentrations versus shear time gives a measure of the degree of desorption caused by shearing (Mikkelsen, 1999).

Mikkelsen (1999) summarised that due to the non-linear dependency of the equilibrium colloid concentration on sludge concentration and shear, strictly, a simple test for floc strength characterisation is not possible. On the other hand, the importance of the colloidal fraction to dewatering suggests that a simple characterisation method would be beneficial for improved understanding of sludge separation properties. Therefore a standardised test was suggested for characterisation of aggregate strength by the "shear sensitivity" parameter in a manner by which the closest possible link to the desorption model is maintained (Mikkelsen 2001; Mikkelsen and Nielsen 2001). The parameter predicts the equilibrium degree of dispersion of sludge standardised with solids content 3.5-4.0 g SS l^{-1} exposed to a shear of G=800 s^{-1} (Root-mean-square velocity gradient).

2.2.5 Settling Properties and Filamentous Microorganisms

In general separation processes at a wastewater treatment plant are directly affected by the settling properties of activated sludge aggregates. Large, regularly shaped and compact aggregates settle fast. Several investigations have been made to correlate the morphology of the flocs to the process parameters by means of microscopy (Eikelboom and Van Buijsen, 1983; Jenkins *et al.*, 1993).

Flocs with good sedimentation properties are generally thought to be made up of floc-forming bacteria and a moderate number of filamentous bacteria (Wilén, 1999) Separation problems are often related to excessive growth of certain filamentous microorganisms. Filamentous bacteria can cause sludge bulking by lowering significantly the floc density.

2.2.5.1 Significance of Filamentous Microorganisms

Usually bulking sludge (Sludge Volume Index (SVI) >150 ml/g) is related to the excess growth of filamentous microorganisms. Due to this widespread problem an enormous amount of research has been carried out in the field of bulking sludge. Beside traditional determination using the identification scheme of Eikelboom and Van Buijsen (1983), recently molecular methods have been introduced to identify and describe the diverse group of filamentous bacteria (Wagner *et al.*, 1994a; Kanagawa *et al.*, 2000). The various filamentous microorganisms can grow in dissimilar forms such as rigid, straight or coiled. More than 30 different filamentous morphotypes have been observed in domestic WWTPs and approximately 40 additional morphotypes were recently described in industrial activated sludge plants (Kämpfer and Wagner, 2002; Eikelboom and Geurkink, in press).

According to Jenkins (1993) the factors which influence the formation of bulking sludge can be divided into general and specific factors. The general factors are sludge age, aeration basin configuration, reactor and settler design. Among others specific factors are dissolved oxygen (DO) concentration, pH, nature and biodegradability of the organic substances, nutrients concentration and temperature. One way to change general factors for bacterial growth is to install selectors (Rensink and Donker, 1991; Jenkins, 1993). The selectors can promote the growth of floc-forming and suppress the growth of some filamentous bacteria (Chudoba *et al.*, 1973a; b; 1974; Wanner, 1993).

In a selector, the food-to-microorganisms (F/M) ratio is high or the conditions are anoxic, which results in a disadvantage for the growth of several types of filamentous bacteria. At low substrate conditions filaments have a competitive advantage and grow faster than floc forming bacteria. This principle is known as the kinetic selection theory (Chudoba and Pujol, 1994). Furthermore some filamentous bacteria are unable to use nitrate as an electron acceptor or to take up substrate during anaerobic conditions. Anoxic and anaerobic selectors may be used to promote the growth of floc forming bacteria. Especially in biological nitrogen and phosphorus removal plants these filamentous organisms are normally not present, because their growth is limited. This is known as metabolic selection (Wanner, 1993).

It has been suggested that the ability to accumulate substrate is a key factor in the kinetic selection of bacteria in an activated sludge system (Chudoba, 1985). The microorganisms which take up most substrate, in the selector or in the inlet part of the aeration tank, will dominate. Floc formers seem to have a greater ability to store substrates than filamentous microorganisms (Majone *et al.*, 1999). Information about maximum growth rates and half-saturation constants for various substrates can be used to predict which bacteria have an advantage over others (Slijkhuis, 1983; Lau *et al.*, 1984). The essential difficulty is still the determination of the limiting factor.

2.2.5.2 Growth Promoting Factors and Practical Experience

Five factors that have been found to promote the growth of some filamentous bacteria include:

- Readily degradable compounds as saccharides, alcohols, low fatty acids, long chain fatty acids and amino acids (Wanner, 1993; Jenkins, 1992);
- Inorganic sulphur compounds (Echeverría *et al.*, 1992);
- Nutrient depletion (Horan and Shanmugan, 1986; Ericsson and Eriksson, 1988; Echeverría *et al.*, 1993);
- Low temperature (Knoop and Kunst, 1998).

Some filamentous bacteria can grow in a wide range of sludge ages while others grow in a narrow range. In plants operated with very long sludge ages, typical of nitrifying-denitrifying activates sludge systems, a certain type of filamentous has been identified: a low F/M filamentous bacteria (Gabb *et al.*, 1991). Although metabolic selection might repress some filamentous organisms in many treatment plants for biological nitrogen and phosphorus removal serious problems with bulking sludge and foaming have been experienced, due mainly to the filamentous bacterium *Microthrix parvicella* (e.g. Eikelboom and Andreasen, 1995; Andreasen and Sigvardsen, 1996; Knoop and Kunst, 1998; Wanner *et al.*, 1998). Nielsen and co-workers (in press) investigated the *in situ* physiology of *M. parvicella* by using microautoradiography (MAR) and provided an interesting hypothesis for the competitive advantage of *M. parvicella* in nutrient removal plants. Their studies showed that in contrast to most other activated sludge bacteria, *M. parvicella,* is capable to take up and store long-chain fatty acids under anaerobic conditions and subsequently metabolize them under aerobic conditions.

The understanding of the microbial community structure in WWTPs, including filamentous morphotypes, continues to advance rapidly. The development of novel molecular-biological techniques (e.g. Lee *et al.*, 1999) for *in situ* analyses has generated an opportunity for investigating the ecophysiology of microorganisms (Nielsen *et al.*, in press). These techniques should help to identify the links between microbial community composition, function and process stability (Wagner and Loy, 2002)

2.3 Flocculation and Floc Formation

Although already mentioned in the context of previous sections this chapter especially deals with the phenomenon of flocculation and the related sludge floc formation. In this study flocculation is primarily defined as an initial step which is necessary for the formation of sludge flocs or granules. However, the formation of sludge flocs and granules is not only influenced by physico-chemical processes in the initial step. Flocculation is dominated by

physico-chemical forces and reactions while sludge floc/granula formation is additionally influenced by the microbiological environment. Both, flocculation and floc formation are complex and decisive steps in the activated sludge process. In the following important floc models are summarised.

A lot of species easily form aggregates (or biofilms) which depends on the species' environmental situation (Wingender *et al.*, 1999). In natural aquatic systems the majority of microorganisms is organised in flocs (or biofilms). Floc formation is a common phenomenon in natural systems and has been investigated for many years (e.g. Bratby, 1980; Unz, 1987; Gregory, 1989; Mozes *et al.*, 1989; Eriksson and Alm, 1991; Wahlberg, 1992; Urbain *et al.*, 1993; Higgins and Novak, 1997c).

Although the parameters influencing the flocculation and moreover the floc formation of activated sludge are to some extent known, their relative significance is still largely unknown. Why flocculation (and the related floc formation) mechanisms cannot easily be schematised is related to different parameters as the following:

- Influence of porosity and shape of the primary particles
- Viscosity changes due to surface active compounds
- Influence of heterodispersity to the flocculation
- Hydrodynamic parameters

Flocculation is influenced by various chemico-physical parameters, such as ionic strength, pH and amount of organic compounds.

2.3.1 Interactions between Surfaces

Bacterial adhesion to surfaces is a large extent based on physico-chemical interactions similar to those governing the interactions of non-living colloids (Rutter & Vincent, 1984; Krekeler *et al.,* 1989). Mikkelsen (1999) summarised interactions between surfaces. Considering sludge as a collection of colloids, their interactions can be expected to depend on a number of forces including both DLVO[2] forces (*van der Waals interactions* and *electrostatic repulsion*) and non-DLVO forces (Unz, 1987; Gregory, 1989; Zita and Hermansson, 1997a;b). Non-DLVO forces are *hydrophobic interaction*, *polymer entanglement*, *polymer bridging*, *hydration forces*, *steric forces* and *specific interactions*. In the DLVO theory, the van der Waals attraction and electrical double layer repulsion are assumed to be additive and combined to give the total Gibbs interaction energy between particles as a function of the separation distance (Zita and Hermansson, 1994). Taking the theoretical considerations into account flocculation can be initiated by dissolved polymers which have to adsorb to the surface of colloids or microflocs.

[2] The theories of Derjaguin and Landau (1941) and Vervey and Overbeek (1948) introduced the fundamental idea that the understanding of complex colloidal phenomenology could be based on the concept of long-range forces, both attractive and repulsive, acting between assemblies of atoms or molecules.

2.3.2 Conceptual Floc Model

The flocculation process is influenced by various types of interactions and several published studies deal with flocculation properties of bacteria isolated from activated sludge (Singh and Vincent, 1987; Kurki *et al.*, 1989; Jorand *et al.*, 1994; Zita and Hermansson, 1997a; b). Mechanisms responsible for pure culture flocculation vary for different species and parallels to flocculation processes in activated sludge can be drawn, but have to be discussed. Several conceptual models for the mechanisms of flocculation have been put forward and were summarised by Wilén (1999):

2.3.2.1 The Polymer Bridging Model

Different models stress the significance of extracellular polymeric substances and of charge neutralising ions. Both seem to decide the floc stability. Flocs contain EPS which act as ion exchange resins, attracting ions and molecules that contribute to the formation of dense flocs or biofilms. (Fowler, 1988; Wingender et al., 1999; Flemming and Wingender, 2000). Important EPS properties are the molecular weight, charge density and the ratio of hydrophobicity to hydrophilicity. EPS is reported to contribute to the formation of dense aggregates (Wingender et al., 1999; Flemming and Wingender, 2000).

In the polymer bridging model (Pavoni, 1972; Eriksson *et al.*, 1992) it is suggested, that the bacteria excrete EPS which join the cells together by means of divalent cations such as Ca^{2+} and Mg^{2+}. The EPS, composed of high-molecular compounds, can form bridges between several cells (steric interaction). Furthermore, EPS contain functional groups that are primarily anionic and non-ionic in the neutral pH range. Divalent cations bind electrostatically to the negatively charged functional groups to generate strong bridges between floc components.

Mikkelsen (1999; 2001) studied EPS in relation to floc structure and stated, that an increased amount of EPS in activated sludge flocs stabilises the flocs regarding shear forces. It was suggested that the physical entanglement of the EPS is more important than DLVO-type interactions for the floc structure, which is in contrary to earlier findings (Mikkelsen *et al.*, 1996). The results indicate that it is necessary to distinguish between long term and short term changes in floc structure. When various sludge are compared, the flocs with the higher EPS content should be less sensitive towards shear stress.

Although it is seems inconsistent that micro-organisms produce more EPS if the loading is lower, this might be explained by the following approach. As explained in section 2.4.2 bacterial EPS protects bacteria e.g. from desiccation and is used as an enzyme pool (Flemming and Windender, 2000). Low loading conditions as a possible stress parameter could therefore force the bacteria to produce EPS. Furthermore this would explain that the outer surface of flocs, which mainly contains younger cells, has less EPS than the interior of flocs. Floc components in outer zones are therefore weakly bound due to limited amount of

EPS. Cells in the inner part of the aggregate are embedded as agglomerates in a strong EPS matrix, hence the cells at the surface are more sensitive to shearing effects (Pavoni *et al.*, 1971).

2.3.2.2 The Filamentous Backbone Model

In the filamentous backbone model (Parker *et al.*, 1971; Sezgin *et al.*, 1978) it is suggested, that filamentous bacteria form a backbone onto which EPS producing bacteria can attach themselves. It has also been proposed that the filaments give rise to stronger flocs (Parker *et al.*, 1971, 1972). However, the filaments are not believed to affect the attachment of other floc components to the flocs. Nowadays it is recognised that filamentous bacteria are not a prerequisite for flocculation.

2.3.2.3 The Colloidal Model

The colloidal interaction model is based on the idea, that the interactions between floc components can be described by the DLVO theory for colloidal stability (Zita and Hermansson, 1994), developed independently by Deryagin and Landau (1941) and Verwey and Overbeck (1948). The DLVO theory has also been shown to describe the interactions involved in bacterial attachment to surfaces (e.g. Van Loosdrecht *et al.*, 1987a; Fowler, 1988; Mozes *et al.*, 1989; Van Loosdrecht *et al.*, 1990; Rijnaarts *et al.*, 1995). Not only single bacteria but also larger floc constituents can be described as colloids. Colloidal systems include particles in the size interval 1 nm-1µm (Shaw, 1992). These particles are affected by Brownian motion: they are not settleable. Larger particles are influenced more by gravity in the particle interaction. In practice, however, particles up to 100 µm can be described as colloids (Gregory, 1989), although they settle.

In this model for colloidal stability, the degree of interactions, depends on the surface potential and on the thickness of the electrical double layers. Negative surfaces are surrounded by positive charged ions, which leads to repulsive *electrostatic interactions* between approaching surfaces. Most common interactions between colloids are the *van der Waals attraction* and *electrical repulsion*. The *van der Waals attraction* forces are determined basically by the separation distance between the particles, the geometry of the system, and the Hamaker`s constant (Shaw, 1992). The Hamaker's constant depends on the hydrophobicity of the interacting compounds (Van Loosdrecht *et al.*, 1990). If the electrolyte concentration is high or if there are polyvalent counter-ions present, the electrostatic repulsion is reduced and bacteria and other floc particles can adhere easier to each other (Shaw, 1992).

Additional ideas for a model with three structural levels were suggested by Jorand *et al.* (1995). There are different levels of microflocs: primary particles (2.5 µm) and secondary particles (bacterial colonies: 13 µm) that are linked together by EPS to form tertiary

structures. This structured aggregates have according to the model a mean diameter of 125 µm. A further improved model was published by Keiding and Nielsen (1997). The authors describe flocs as being a cloud of organic macromolecules and single bacteria attached by very weak forces in contrast to a more rigid backbone consisting of fibers, filaments and bacterial colonies embedded in EPS.

2.3.3 Hydrophobic Interactions

Steric and DLVO-type interactions as well as polymer bridging are involved in flocculation processes, but hydrophobic interactions also take part. Charged colloids are hydrophilic and soluble in water, whereas non-polar colloids are not or less soluble in water, because of their hydrophobic surface. In aqueous solutions, molecules with hydrophobic parts can be associated in such a way that the contact with the water phase is minimised, this is called hydrophobic interaction (Gregory, 1989).

Several studies could show that the degree of hydrophobicity of bacteria influences the cell adhesion (Rosenberg and Kjelleberg, 1986; Van Loosdrecht *et al.*, 1987b; Mozes *et al.*, 1989; Van Loosdrecht *et al.*, 1990). There are diverse factors affecting the cell surface hydrophobicity: (1) growth conditions in general (Hazen *et al.*, 1986; Van Loosdrecht *et al.*, 1987b; Grotenhuis *et al.*, 1992) (2) starvation (Kjelleberg and Hermansson, 1984); (3) growth phase (Hazen *et al.*, 1986); and (4) temperature (Blanco *et al.*, 1997). Oxygen limitation can reduce the hydrophobicity of bacteria from activated sludge (Palmgren *et al.*, 1998).

Urbain *et al.* (1993) proposed that hydrophobic interaction inside flocs is important for the flocculation process. The co-existence of hydrophilic and hydrophobic bacteria in activated sludge was observed by Jorand *et al.* (1994), who additionally could show that the hydrophobic properties of the related bacteria can vary during different growth phase. Zita and Hermansson (1997a; b) collected further data for the significance of bacterial adhesion for flocculation. Olofsson *et al.* (1998) studied the attachment of hydrophobic and hydrophilic bacteria to activated sludge flocs. High degrees of cell surface hydrophobicity correlated well with adhesion to flocs. Recently Nielsen *et al.* (2001) investigated the surface hydrophobicity of different types of bacteria in activated sludge under *in situ* conditions by following the adhesion of fluorescent micro-spheres with defined surface properties to bacterial surfaces (MAC-method: micro-spheres adhesion to cells). This technique was combined with identification of the bacteria with fluorescence *in situ* hybridisation with rRNA-targeted oligonucleotides (FISH). The dominating types of filamentous bacteria showed significant differences in surface hydrophobicity.

It can be summarised that there are some facts known about hydrophobicity regarding the flocculation and floc formation of activated sludge, but interpretation of the results remains difficult. New combinations of methods to determine *in situ* the cell surface hydrophobicity (MAC-FISH) are necessary for an improved understanding.

2.4 Microbial Aggregates in Wastewater Treatment

2.4.1 Bacterial Aggregation

Aggregates were observed for bacteria, yeast, cellular slime molds, filamentous fungi and algae (Calleja, 1984). The microbial capability to aggregate was investigated by several authors. Yet many questions remain open, however, facts about the mechanism, and related environmental conditions are known. There are reports that indicate that aggregation is inducible.

The presence of divalent metal ions, which could act as a bridge between negatively charged groups on cell surfaces, have proved to be important in the aggregation process. It was demonstrated that the concentration of calcium ions in the range of 40-100 mg l^{-1} in the waste water stream enhanced the rate of sludge aggregation (de Zeeuw, 1981).

Eighmy *et al.* (1983) demonstrated that the process of bacterial adhesion was related to the negative surface charge density and to the relative hydrophobicity/hydrophility at the surface. When inert particles are present, the aggregation depends significantly on the physical properties of surfaces (de Vocht *et al.*, 1983; Switzenbaum *et al.*, 1987; Huysman *et al.*, 1983; Mozes *et al.*, 1987; Van Loosdrecht *et al.*, 1987).

Anaerobic sewage bacteria were induced to aggregate with increasing concentrations of Fe^{3+}, Al^{3+}, Ba^{2+}, Mg^{2+} and Ca^{2+}. An increase in the tendency to aggregate was correlated with a reduction in the negative cell surface charge (Kosaric, 1987).

Kosaric and Blaszczyk (1990) stated that the microbial capability to aggregate seems to be inducible. The aggregation process is influenced by environmental conditions, which can cause a change in physiology so that the microbes change from a dispersed to an aggregated state.

2.4.2 Anaerobic and Anoxic Granular Sludge

Granulation of anaerobic sludge has been well documented, especially in UASB reactors (e.g. Lettinga *et al.*, 1980; Kosaric and Blasczyzk, 1990). The concept behind upflow sludge blanket reactors is that mechanical agitation and sludge recirculation are kept at a minimum. Finely dispersed poorly flocculating matter is washed out of the reactor, as it interferes with the sludge flocculation.

Granulation is also known to occur in some denitrification reactors, anaerobic sequencing batch reactors (ASBR), and biofilm airlift suspension reactors (BAS). Granular sludge demonstrates good settling properties, good solid-liquid separation, high biomass retention, high activity, and an ability to withstand high loading rates (Kosaric and Blasczyzk, 1990; Kosaric *et al.*, 1990).

Spherical granules allow for the maximal microorganism to space ratio (Guiot *et al.*, 1992). Granules have been defined as spherical biofilm (Grotenhuis *et al.,* 1991b). Granular methanogenic sludge can remain well conserved under unfed conditions for several years (Kosaric and Blasczyzk, 1990). In accordance with Stoke's Law, their excellent settling is a result of their size (Guiot *et al.*, 1992). In the following, experimental results leading to the formation of anaerobic granules are described.

2.4.2.1 Batch Reactor versus Upflow Reactor

Dolfing *et al.* (1987) studied the effects of different substrates on granules in both upflow and batch reactors. Using UASB reactors, they found that ethanol as the carbon source led to the best granule formation. Different other substrates resulted in a comparatively higher amount of filamentous bacteria and a higher SVI. The use of formate resulted in the disintegration of the granules. They concluded through parallel experiments that the formation of granules is initially a biological phenomenon, influenced by the choice of substrates. This could mean that the biological processes dominate the granula formation, e.g. by the production of EPS which influence the hydrophobicity or other surface relevant properties. Moreover the microbial community composition seems to be influenced by the carbon sources.

Wirtz and Dague (1996) studied possibilities for the enhancement of granulation in anaerobic sequencing batch reactors. The addition of three additives (powdered activated carbon, granular activated carbon (GAC) and cationic polymers) had positive results. Using these additives granulation was up to three months faster, respectively, than in the control reactor. Enhancers such as GAC offer a support matrix for the bacteria.

In addition to enhancers, Wirtz and Dague (1996) stated that a short hydraulic retention time and a relatively high organic loading rates are beneficial to granulation. Even the control reactor (without added enhancers) had granulation in only 4-5 months, in contrast to earlier experiments by Wirtz and Dague where granulation took 10 months.

2.4.2.2 Granule Formation and Structure

There are several theories concerning granule formation. It is generally thought that the upflow velocity in a UASB creates a selective pressure. Microorganisms are washed out or bind together and form easily settleable granules (Guiot *et al.,* 1992).

Kosaric and Blasczyzk (1990) noted that the velocity at the inlet port and the port configuration have a strong influence on granulation. Other sources point to the methanogenic microorganisms found in granules or EPS as being the cause of pelletisation. *Methanosarcina* and *Methanosaeta*, commonly found in anaerobic sludge granules, both exhibit natural tendencies to aggregate and are thought to aid in pelletisation (Kosaric and Blasczyzk, 1990; Fang *et al.*, 1994).

The high activity of granules is attributed to the proximity and interaction of various groups of microorganisms (Wirtz and Dague, 1996; Guiot *et al.*, 1992). Young anaerobic granules usually have centres composed of *Methanosaeta* surrounded by *Methanosarcina* (Kosaric and Blasczyzk, 1990).

Giuot *et al.* (1992) also described stratified structure of granules from a mesophilic reactor as having three layers, each with distinguished morphotypes (*Methanosaeta, Methanobrevibacte, Methanococcales*-like organisms and *Methanosprillum*-like filaments).

At low loading rates, anaerobic granules may develop hollow cores due to substrate deficiency (Kosaric and Blasczyzk, 1990; Grotenhuis *et al.*, 1991a; Guiot *et al.*, 1992). This decreases the density, inhibiting their settling and causing washouts. Large aggregates may also have hollow cores resulting from substrate limitation. Similarly, the hollow cores can weaken the structure of large granules, causing their disintegration.

2.4.2.3 Substrate Composition and Loading Rate Effects on Granules

A correlation has been noted between the load applied and the granule strength (Quarmby and Forster, 1995) and size (Grotenhuis *et al.*, 1991a; Fang *et al.*, 1994). Wastewater composition and volumetric loading strongly influence sludge granulation (Kosaric and Blasczyzk, 1990).

Grotenhuis *et al.* (1991a) studied the effect of substrate concentration on methanogenic granule size distribution. The methanogenic activity was found to be directly proportional to influent concentrations. Granule size increased until substrate limitation occurs in the centre of the granule. When this occurred, the substrate limited biomass in the centre begins to decay. This weakened the granule making it susceptible to shear forces. (Kosaric and Blasczyzk, 1990; Grotenhuis *et al.*, 1991a).

Van der Hoek (1987) stated that the stability of aggregates in denitrifying granular sludge in USB reactors is largely dependent on what type of substrate is used.

Resuming anaerobic granulation studies it can be stated that there are several hypothesis for the formation, but the exact mechanism has not been determined yet. It is a biological phenomenon that seems to be directly influenced by the choice of substrate, the organic load and the hydraulic retention time. The quantity of facts collected in many studies clearly indicate that anaerobic granular formation can be regarded as a result of selective pressure. Some investigations point to the methanogenic microorganisms found in granules or to EPS as being the cause of pelletisation. *Methanosarcina* and *Methanosaeta*, commonly found in anaerobic sludge granules, both exhibit natural tendencies to aggregate and are thought to aid in pelletisation.

2.4.3 Aerobic Granular Sludge

Mishima & Nakamura (1991) and Shin *et al.* (1992) were among the first ones to report that it was possible to develop aerobic granules in an upflow reactor.

Laboratory scale sequencing batch reactors were successfully used by Morgenroth *et al.* (1997) to cultivate aerobic granular sludge. A substrate loading rate of 7.5 kg COD $(m^3\ d)^{-1}$ was applied which led to granules with an average diameter of 3.3 mm. To enhance the growth of granular sludge the SBR was operated with very short sedimentation and decant phases resulting in an increased washout of slow settling biomass. Aerobic granules became the dominant aggregates in the reactor. The authors assumed that granular structure was in the beginning dominated by filamentous yeast cells (*Geotrichum sp*.). It should be noted that the authors used plating techniques and contamination with yeast cells cannot be excluded.

Generally fungi and yeast are known to form easily mycelial pellets which are not washed out, because of their good settling properties. Based on microscopic observations a possible mechanism for the granule formation was proposed. The identified yeast cells formed a backbone structure, which was believed to be an important and characteristic step in the granule formation. First yeast-pellets formed and possibly due to hydrodynamic shear stress their shape became round. These pellets grew up to 6 mm in diameter, but were not stable and broke up. The embedded bacterial colonies inside the aggregates were suggested to be the final core of the afterwards growing granules, which had an average diameter of 3.3 mm.

Zhu and Chunxin (1999) cultivated granular activated sludge using an alternating anaerobic/aerobic process. They reported the cultivation and physico-chemical characteristics of granular activated sludge in a single alternation of anaerobic/aerobic (AAA) process. It was shown that under certain conditions, the granular activated sludge was formed in the reactor. Characteristics of the granular sludge were: 0.2~1.5 mm in diameter, wet specific weight about 1.007 g/cm^3. For a domestic sewage of COD 370~550 mg/L and P concentration of 9.8~13.6 mg/L , the COD and P removal efficiencies reached more than 90 %.

Dangcong *et al.* (1999) reported the cultivation of aerobic granular sludge in a laboratory SBR using synthetic wastewater (carbon source was acetate) and controlling the DO at low concentration (0.7-1.0 mg/l). Granula shape was nearly spherical (0.3-0.5 mm in diameter) with a very defined and smooth surface. The granular sludge showed good settleability (SVI between 80-100 ml g^{-1}) and high COD removal and nitrification activities. After 20 days of operation the SVI decreased and filamentous bacteria in the sludge disappeared. Flocculent sludge changed gradually to granular sludge. One month was necessary to cultivate dominant and stable granular sludge. Granula structure was characterised to be similar to anoxic granula (Krachtovil *et al.*, 1996). Microscopic observation showed rod-like bacteria, but no filamentous bacteria.

Beun and co-workers (1999b; 2001) investigated the formation and cultivation of aerobic heterotrophic granular sludge in a sequencing batch reactor using synthetic wastewater

(carbon source was acetate). Further the authors observed spontaneous development of aerobic granules in an airlift reactor. In the reactors biomass normally grew as a biofilm on small suspended basalt particles. The laboratory airlift reactor was run in sequencing batch mode, hence the sludge was exposed to high shear stress. An efficient wash-out of slow settling flocs, by using a short settling time of 2 min., led to dense granules. Granules with a diameter of 1.0 mm could be formed. Process conditions were strictly aerobic and total cycle time was 3 hours. Beun assumed that microorganisms prefer to grow in suspension over growing in flocs, biofilm or granules and therefore granule formation occurs only when bacteria are forced to do so by means of proper environmental conditions. A short settling time was chosen, combined with high and tall reactors particles with a high settling velocity could be effectively selected and finally granular sludge could be cultivated.

Although granula grow in suspension, they might be compared with biofilm grown on carrier particles, since the process conditions and therefore the growth conditions of the bacterial cells are similar. If granula are a special case of biofilm growth, the formation, growth and shape of the granules would be mainly influenced by the substrate loading rate on the one hand and by the shear forces on the other hand (Van Loosdrecht *et al.*, 1995).

In a recent study granulation in an anoxic/anaerobic-aerobic SBR of laboratory scale was described. The authors (Dülükgürgen., unpublished) used synthetic wastewater with acetate as carbon source and an average influent concentration of 270-300 mg COD l^{-1}, 20-25 mg l^{-1} PO_4-P and 35-40 mg l^{-1} NH_4-N. The process was characterised by a cycle time of 6 hours, 1 hour of continuous filling, totally 2 hours of mixed react and 3 hours of aeration. 30 min. settling and 30 min. draw at a sludge retention time of 8 days. Flocs were the only sludge aggregates within the first 3 months of operation. Settling time was shortened to 15 min. and 3 weeks later small granules appeared in the reactor. Light microscopic observations indicated the presence of the species Zoogloea, but no filamentous bacteria could be identified.

An industrial airlift system, which produced granular sludge was reported (Mulder and Frijters, 2000). Biomass concentrations of up to 30 g VSS l^{-1} and conversion rates of around 5 kg COD $(m^3 \text{ day})^{-1}$ could be achieved. Several industrial effluents could be successfully treated with a high COD and nitrogen removal efficiency.

I a recent review (Liu and Tay, 2002) the essential role of hydrodynamic shear forces in the formation of granular sludge was discussed. Aerobic granulation is regarded as a self-immobilisation process. The authors conclude that shear forces had significant influences on the structure of granular sludge and the production of EPS. Tay *et al.* (in press) could show that increasing hydrodynamic shear forces resulted in aerobic granules with a significant higher density.

Zhu and Wilderer (2003) investigated the metabolic activity of aerobic granular sludge which was cultivated in laboratory SBR. The oxygen consumption rate was used as an indicator to evaluate the metabolic activity. The results revealed that the size, colour and the settling

characteristics of the granular sludge did hardly change after seven weeks of anaerobic storing. It took less than a week to regain full activity of the granular sludge.

Aerobic granular sludge showed not only storability for weeks, moreover Tay et al. (2002) reported the presence of anaerobic bacteria within aerobic granules. Especially enhanced nutrient removal can profit of a diverse microbial community composition. In fact, the application of aerobic granules has been studied for nitrogen and phosphorus removal (Liu *et al.*, 2003; Zhu and Wilderer, 2003)

McSwain *et al.* (in preparation) concluded in their study using three parallel SBR that a high feast-famine ratio, or pulse feeding provided by dump fill in the reactors was necessary for the formation of compact granules. An intermittent feeding affected the selection and growth of floc-forming and filamentous organisms. This influenced the structure of the aerobic granules.

Regarding the mentioned studies it can be stated that aerobic granules grow in suspended way similar as activated sludge flocs, but might be classified between activated sludge and biofilms, because there are similarities to biofilms which grow on small sized support materials. The granules show good settling properties and can be stored for weeks. Cultivation of aerobic granular sludge is observed in laboratory reactors operated in sequencing batch mode. Granulation is not strictly restricted to certain specific species. The contribution of operation conditions, reactor configuration and microbial or genetic factors to the granulation is unclear. The granulation seems to be inducible (Liu and Tay, 2002) and takes as a rule between 1 and 4 weeks. There are some reports (Morgenroth *et al.*, 1997; Beun *et al.*, 1999a) indicating that a short settling phase in the SBR is necessary to initialise the granulation, although other investigations contradict this hypothesis. In comparison to anaerobic granules the microbial community is poorly characterised and information about dominant bacterial groups and the significance of filamentous organisms are missing. Hence the key process parameters for the formation of aerobic granular sludge are not yet defined.

2.4.4 Comparing Different Granular Sludges

Although granular sludge, cultivated under anaerobic, anoxic or aerobic conditions, is obviously very heterogeneously composed some general conclusions can be drawn. All granular sludge aggregates are formed by microbial organisms, and in anaerobic granular sludge dominating organisms could be identified. Function and interaction of microorganisms are only to some extend investigated and many questions are open. Generally every microbial population is dependent on the environmental conditions. Hence anaerobic and aerobic granular sludge will naturally have a completely different distribution of organisms, although anoxic or anaerobic zones might be found within aerobic granules. This could mean that granulation mechanisms for anaerobic and aerobic granular sludge are different.

There have been many studies to characterise anaerobic and anoxic granular formation, structure and functions, but it was not possible to link the granulation process to one single

key parameter or a set of key parameters. Many facts were collected and the formation as well as the microbial structure could be characterised by a set of important parameters. The first investigations regarding aerobic granular sludge were based on the conclusions drawn by anaerobic granular studies. For anaerobic granular sludge short HRT and relatively high OLR seem to be beneficial for granulation. A low HRT causes poor-settling biomass to wash out while the high OLR ensures sufficient new biomass growth, which is balanced by moderate shear stress.

In conclusion it can be summarised that special environmental conditions are necessary to promote granula formation. Due to the fact that many organisms have the ability to aggregate and to form granula, granules can be cultivated in anaerobic, anoxic and aerobic conditions. At the moment the granulation mechanism seems not to be generally predictable, although useful hypothesis and guidelines have been worked out in anaerobic sludge studies. The granules once formed are stable without feeding even for months. The formation and stability of those biological aggregates seems to be in general influenced by the loading and hydrodynamic conditions. A structural zonation could be detected for anaerobic granules and lysis within the granules resulted in hollowing of aggregates. For aerobic granular sludge some data regarding the formation is available, but questions remain open. Important structural and functional aspects are not yet clearly described, the mechanism of the formation is hardly investigated and data are limited to laboratory investigations. In contrast to anaerobic granular sludge no full scale application of aerobic granular sludge is documented. A comparison between the different sludges is limited.

2.5 *In situ* Techniques

The function of a wastewater treatment plant is determined by the activities and interactions of its microbial community. Thus, information on the identity of microorganisms responsible for specific activities, of interactions between cells of the same or different populations and information on the influence of changing environmental conditions are important for optimising the treatment process. However, classical approaches to investigate those complex ecosystems, e.g. physico-chemical bulk measurements, usually do not lead to a comprehensive picture of the microbial community nor can they resolve complex processes in aggregates (flocs or granula) or biofilms. These habitats are highly stratified systems with steep physico-chemical gradients and organisms adapted to sequential transformations along those gradients (Schramm, 1998). Therefore, sophisticated *in situ* techniques are necessary to identify microorganisms and measure activities at the place of their occurrence or, in other words, to analyse structure and function of biofilms and microbial aggregates on a microscale.

2.5.1 Microsensors

In situ structure and function analysis are prerequisites for both understanding the complex network of microbial populations and processes inside biofilms, activated sludge flocs and granules. One tool to investigate microbial ecology are microsensors, which are minimally invasive instruments to measure gradients of important metabolites and parameters with high spatial resolution. The gradients can also be used to evaluate the zonation and rates of the measured processes (Kühl and Revsbech, 2000).

Bacterial aggregates, flocculated and granulated sludge, both might be seen as kind of "mobilised" biofilms (Van Loosdrecht *et al.*, 1995). Although there exists no support material, they share most other characteristics with typical biofilms, since the aggregates consist of cell clusters embedded in EPS with a irregular structure and they usually are surrounded by a boundary layer that allows mainly diffusional transport of solutes. Hence often chemical gradients and spatially distinct microenvironments are developed (de Beer *et al.*, 1998; de Beer *et al.*, 1993a; Lens *et al.*, 1995; Ploug *et al.*, 1997). Therefore, the same high resolution *in situ* techniques are needed for the structure and function analysis of aggregates as they are needed for biofilm investigations.

The determination of microenvironments and *in situ* activities inside biofilms and sludge aggregates requires tools with high spatial and temporal resolution. Microsensors with a tip diameter of 1-50 µm have thus been developed and applied in microbial mats and biofilms to measure O_2, pH, CO_2, H_2, H_2S, S^{2-}, NH_4^+, NO_2^-, NO_3^- and other parameters. Important disadvantages of microsensors are their limited resolution and the local disturbance. Spatial profiles are the result of single point measurements (e.g. Revsbech and Jørgensen, 1986; Schramm *et al.*, 1999; Kühl and Revsbech, 2000).

Microsensors rely on either electrochemical or optical principles (Kühl and Revsbech, 2000). The electrochemical sensors can be divided in three groups: amperometric, potentiometric and biosensors. Potentiometric sensors detect an electrical potential difference generated by a charge separation for ions across a membrane. This ion-selective membrane can be either special glass as for the pH electrode or a liquid ion exchanger (LIX) as for LIX microsensors. LIX sensors can be made very small (1µm), but often suffer from a short lifetime and a low selectivity of the membrane (Santegoeds *et al.*, 1998). Examples are LIX sensors for NH_4^+ (de Beer and Van den Heuvel, 1988), NO_2^- (de Beer *et al.*, 1997b) or NO_3^- (de Beer and Sweerts, 1989). Microbiosensors combine catalysts (i.e. enzymes or whole cells) with electrochemical sensors. Whereas fiber-optical microsensors (micro-optodes) are either used to directly measure light distribution in a sample (Kühl *et al.*, 1994), or certain an indicator chemistry at the fiber tip, that changes its luminescence or absorption in response to an analyte. Oxygen (Klimant *et al.*, 1995), pH and temperature optodes have been developed based on that principle.

2.5.2 Microbial Diversity Analysis

By studying microbial communities in biofilms and sludge aggregates many questions about species composition, structure and bacterial distribution as well as the spatial activity can be posed. In this context traditional microbiological techniques and conventional microscopy were not able to answer the demand (Wagner *et al.*, 1993). Molecularbiological techniques, which do not require isolation of bacterial strains are more and more frequently used to detect and characterise bacteria in natural environments. Muyzer and Ramsing (1995) and Wagner and Amann (1997) reviewed the potentials and limitations of different molecularbiological techniques which are nowadays used to characterise microbial communities. A recent publication describes modern scientific methods and their potential in wastewater science and technology (Wilderer *et al.*, 2002).

2.5.2.1 Full Cycle 16S rDNA Approach

The 16S rDNA approach has become the gold standard for the cultivation-independent assessment of bacterial diversity in natural and engineered systems (Amann *et al.*, 1995). The comparative sequence analysis consists of DNA extraction, subsequent PCR amplification of (a fragment of) the 16S rDNA gene using primers targeting regions conserved in the bacterial domain, cloning and sequencing (Figure 3). The phylogenetic affiliation is determined by analyzing the obtained sequences together with adequate reference sequences.

Using premanufactured kits (e.g., for cloning) and automated sequencers it is possible to analyze relatively high clone numbers. It should be checked, if the number of analyzed clones does sufficiently well represent the diversity in the established library. For this purpose, the clones should be grouped into operational taxonomic units (OTUs) according to their 16S rDNA similarities with each other. Once the clones have been assigned to OTUs, rarefaction analyses (Tipper, 1979) or coverage estimates (Giovannoni *et al.*, 1995) should be performed to determine whether the analyzed number of clones represents a sufficient sample (Loy *et al.*, 2002).

After DNA isolation the 16S rDNA gene (present in every microorganism) is amplified with universal primers by PCR. Subsequently, the resulting 16S rDNA mixture is cloned and a clone library with each clone representing the 16S rDNA gene of one single organism is established. Sequence analysis followed by phylogenetic analysis of the respective sequences reveals a phylogenetic position of organisms within the sample. Subsequently, specific oligonucleotide probes for these organisms can be design and used for *in situ* hybridisation of the original sample.

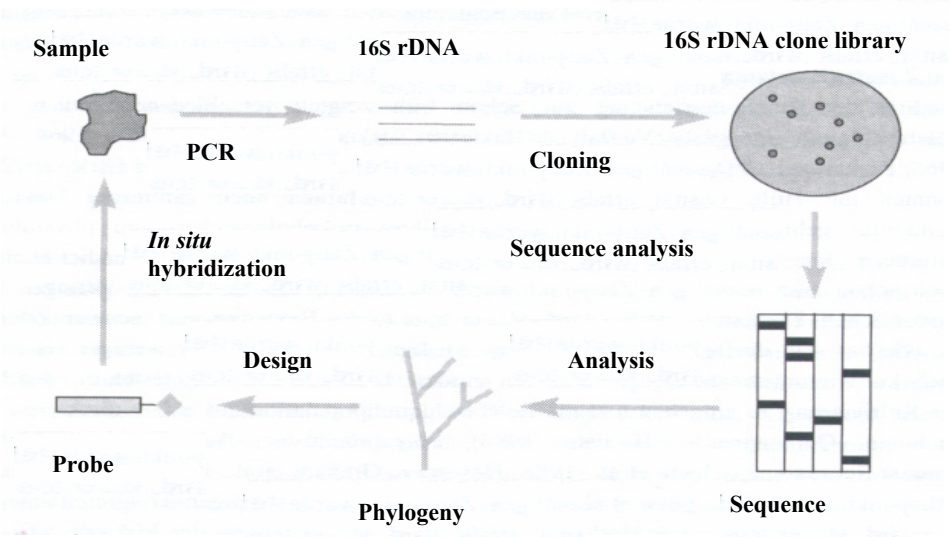


Figure 3. "Full cycle 16S rDNA approach".

2.5.2.2 Ribosomal RNA as Molecular Marker

Ribosomal RNA genes or their transcripts have particular advantages as they are present in all known organisms and it is possible to find general as well as specific target sites for probes or PCR primers. Important is also that they have enough sequence information to be used as a reliable phylogenetic marker and the genes are not transferred horizontally between species.

There are different methods to extract nucleic acids to use the bacterial DNA (or RNA) as target DNA in a PCR to amplify specific genes (for possibilities and limitations of nucleic acid amplification see: Vaneechoutte and Van Eldere, 1997). PCR in general is a procedure for in vitro enzymatic amplification of a specific segment of DNA. Within hours millions of copies can be synthesised using specific oligonucleotide primers and DNA polymerase. Amplification of this magnitude greatly facilitates subsequent analytical procedures.

Another possibility to analyse complex mixtures of microorganisms is to separate PCR products from different organisms by a special kind of electrophoresis, i.e. denaturing gradient gel electrophoresis (DGGE). In DGGE, DNA fragments can be separated to distinguish bands in the separation patterns, which are most likely derived from the predominant species within the communities (Muyzer and Ramsing, 1995).

2.5.2.3 FISH: Fluorescence-*In-Situ*-Hybridisation

Important molecular tools to enumerate particular bacterial populations and to determine their spatial distribution are fluorescent nucleotide probes. The synthetic oligonucleotides are highly specific probes that bind selectively to the desired target sequence. The detection limit depends on which strategy is used to label the oligonucleotide probe as well as on the presence of background "stain" (Muyzer and Ramsing, 1995). As the ribosome concentration in cells is often related to their growth rate, it has been possible to demonstrate a linear

relationship between the average fluorescence intensity per cell volume and the growth rate of the cell culture (Poulsen *et al.*, 1993). *In situ* hybridisation with fluorescent probes has been used to identify unculturable organisms (e.g. Spring *et al.*, 1992), to localise specific bacterial cells in activated sludge (Wagner *et al.*, 1994, Rosselló-Mora *et al.*, 1995), in biofilms and to characterise bacterial isolates.

By using a combination of FISH and CLSM it is possible to get quantitative FISH data on the bacterial community structure. With digital image analysis and semiautomatic quantification protocols specifically stained biovolumes of a target population can be measured. The obtained results cannot directly be compared among different samples. However, by normalizing, which means taking biomass differences into account, intersample comparison is possible (Loy *et al.*, 2002).

Although there are many applications of molecular-biological techniques in microbial ecology still problems have to be solved. One potential disadvantage of FISH is its relatively high detection limit of approximately 10^3 to 10^4 target cells ml^{-1}. Reproducibility and reliability of nucleic acid extraction is important to obtain a real view of the genetic diversity of bacteria in natural samples.

Regarding PCR preferential amplification of target DNAs from some bacteria might occur and chimera molecules could be formed during the amplification process. Often observed problems with *in situ* applications of oligonucleotide probes to natural samples include the following: (1) uneven cell penetration, (2) low signal intensity, (3) high amounts of background auto-fluorescence and in some samples (4) high levels of unspecific staining (Von Wintzingerode *et al.*, 1997)

Lysis of microbial cells from environmental habitats marks a critical step in a PCR-mediated approach. Insufficient or preferential disruption of cells will most likely bias the view of the composition of microbial diversity. Fragmented nucleic acids are sources of artefacts in reverse transcription or PCR amplification experiments and may contribute to the formation of chimeric PCR products (Loy *et al.*, 2002).

The auto-fluorescence of some bacteria or their extracellular polymeric material and the background fluorescence of inorganic particles can be stronger than the probe elicited fluorescence. The use of CLSM is helpful to enhance the specific fluorescent signals and the combined application of multiple fluorescent oligonucleotide probes might increase the detection signal (Muyzer and Ramsing, 1995).

3 HYPOTHESIS

The current knowledge about formation, structure and function of aerobic granular sludge is limited. Key factors affecting the granulation process of aerobic granular sludge remain unclear.

In general, the goals of this study reflect the open questions of chapter 2 about the formation and structure of aerobic granules. The presented work aims at contributing to the understanding of aggregation/granulation in aerobic wastewater systems in general and to the granulation in SBR processes in particular. Additionally, different tests to determine physico-chemical parameters related to structure and properties of aerobic granules are described, e.g. the nutrient removal and the shear sensitivity.

Based on the review of previous research reported in literature the investigations in this dissertation were based on the following hypothesis (1.-5.):

1. **Aerobic granulation is a biological phenomenon. Granulation can be influenced by biological and physico-chemical processes.**

2. **Filamentous organisms play a major role in the formation and structure of granules and especially fungi, build a structural backbone of aerobic granules.**

   The first and second hypothesis are based on the results of Morgenroth *et al.* (1997) and on the general conclusions of Henze *et al.* (1997) who stated that in activated sludge systems different kind of organisms are responsible for the biological processes related to wastewater treatment. The situation at each treatment plant is different and the microbial community composition is directly related to the external conditions.

   Morgenroth *et al.* (1997) reported the presence of filamentous organisms and identified a filamentous yeast in aerobic granules. The results indicated that aerobic granulation is a biological or a combined biological/chemical phenomenon. The authors stated that filamentous organisms play a major role in the formation and structure of the granules.

   In this study lightmicroscopic observations and a detailed molecular analysis of the microbial community composition should be used to detect filamentous organisms and to characterise the aerobic granular sludge. By cryo-embedding and cryo-sectioning of single granules in combination with molecular-biological techniques (PCR, FISH) the dominant organisms and their distribution as well as their activity should be determined.

3. **Granulation is a result of selective pressure on the microbial species distribution. Using SBR, especially a short settling phase and a high volumetric exchange rate in combination with a high shear stress, should be beneficial.**

   The third hypothesis is based on the following conclusions:

On the one hand, in SBR systems the process can be precisely controlled and rather specific environmental conditions can be achieved. The environment is determined by a cyclic and defined change of process conditions. In SBR short settling phases in combination with high volumetric exchange can be useful to concentrate microorganisms which have a good ability to aggregate and build rather fast settling sludge flocs. Flocs and granular sludge with good settling properties can remain in the system while others are washed out.

On the other hand, the activity and diversity of microorganisms are greatly affected by the chemical and physical conditions of their environment (Henze *et al*., 1997; Madigan *et al*., 1997). Microorganisms in SBR systems react to the specific environment. Due to the SBR process the microbial activity can change and/or a shift of the microbial diversity is possible.

If aerobic granulation is a result of selective pressure the granulation should be reproducible using defined process conditions. In parallel SBR experiments single process parameters and the reproducibility of the granulation should be investigated. Laboratory scale SBR should be used under various operating conditions to improve the granulation process and to evaluate the response to transient conditions.

4. **Activated granular sludge settles faster than flocculent sludge due to an increased density.**

   Granular sludge settles very fast and efficiently. Important parameters for the sludge settling are the size and the density of the granules as well as their interaction in the settling phase.

   By determining the buoyant density of aerobic granules in combination with settling experiments using single granules, it should be possible to answer the fourth hypothesis.

5. **An increasing diameter of the granular sludge will lead to a diffusional limitation inside the aggregate. Anoxic and anaerobic zones within granula should be present.**

   Microbial processes are directly related to the concentration of oxygen and of inorganic as well as of organic nutrients. Inside the granula the concentration of oxygen and of nutrients can be lower than at the surface. Micro-sensors can be used to measure *in situ* gradients. Using micro-sensors, information on structural and functional aspects, e.g. the presence of anoxic zones inside the granules, can be obtained.

   In this study micro-sensors for oxygen, NH_4^+, NO_3^- and NO_2^- should be used to investigate micro gradients inside aerobic granula.

4 MATERIALS AND METHODS

4.1 Reactor Set-Up

Mainly, two sequencing batch reactors were operated in parallel. A schematic view of the laboratory sequencing batch reactor set-up is shown in Figure 4 and a picture of one SBR is shown in Figure 5.

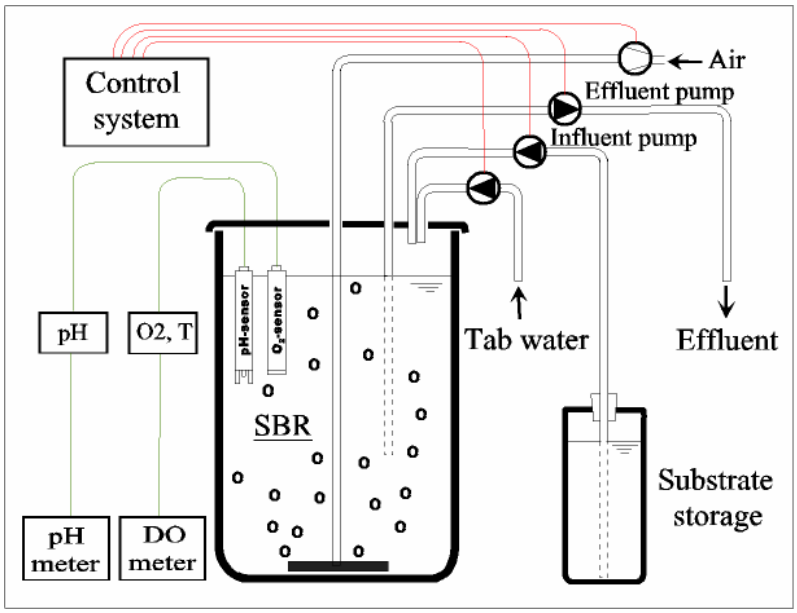


Figure 4. Schematic View of the Lab Scale SBR System

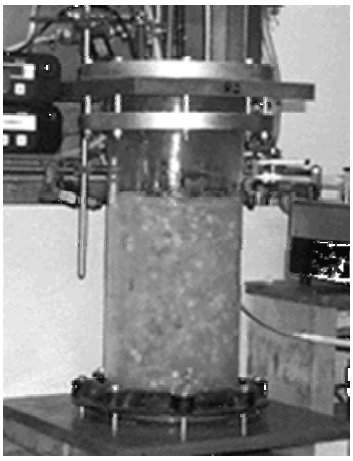


Figure 5. SBR

The influent was composed of tab water and a concentrated substrate solution with various carbon sources. The concentrated substrate solution started to fill 2-3 min. earlier than the tab water stream. The air flow applied for aerating the reactor was in the range of 150-180 l h^{-1}. The physical properties of the reactor are summarised in Table 1.

Table 1. Physical Characteristics of the SBR

Working Volume	10 l
Overall Height of Reactor	0.50 m
Diameter of Reactor	0.30 m
Volumetric Exchange Ratio (VER)	50-75 %
Fill Time Ratio (FTR)	0.04
Air Flow Rate during Aeration	200 l h^{-1}
Superficial Gas Velocity	0.035 m s^{-1}

4.2 Operation

Laboratory reactors were run using different readily biodegradable synthetic wastewaters. The synthetic wastewaters contained all essential organic and inorganic components to promote microbial growth. A low to medium COD level was chosen, which can be in As carbon sources acetate, sucrose, caproic acid, glycerol and a mixture of glucose and peptone were applied. During 30 months of experiments operating conditions of the SBR were varied in 15 relevant experimental phases as summarised in Table 2.

After each experimental period the reactors were inoculated with activated sludge (2.5-3 l/reactor) from the municipal treatment plant of the city of Garching.

Table 2. Different Experimental Periods

Period	Process Characteristics		Influent Characteristics	
1	Filling time (min.)	7	Raw municipal waste water	
	Settling time (min.)	1	COD (mg l^{-1})	200-250
	Total cycle time (min.)	240	Substrate loading (kg (m^3 d) $^{-1}$)	0.7
	Sludge age (days)	15		
	Volumetric exchange (%)	50		
	Anaerobic/aerobic	0/1[3]		
2	Filling time (min.)	7	Carbon source	Acetate
	Settling time (min.)	1	COD (mg l^{-1})	1200
	Total cycle time (min.)	240	Substrate loading (kg (m^3 d) $^{-1}$)	3.6
	Sludge age (days)	15		
	Volumetric exchange (%)	50		
	Anaerobic/aerobic	0/1[3]		
3	Filling time (min.)	7	Carbon source	Caproic acid
	Settling time (min.)	1		Glycerol
	Total cycle time (min.)	240		D-Sucrose
	Sludge age (days)	15	COD (mg l^{-1})	400
	Volumetric exchange (%)	50	Substrate loading (kg (m^3 d) $^{-1}$)	1.2
	Anaerobic/aerobic	0/1[3]		
4	Filling time (min.)	7	Carbon source	Caproic acid
	Settling time (min.)	2		Glycerol
	Total cycle time (min.)	240		D-Sucrose
	Sludge age (days)	15	COD (mg l^{-1})	400
	Volumetric exchange (%)	60	Substrate loading (kg (m^3 d) $^{-1}$)	1.44
	Anaerobic/aerobic	0/1[3]		
5	Filling time (min.)	24	Carbon source	Caproic acid
	Settling time (min.)	4		Glycerol
	Total cycle time (min.)	240	COD (mg l^{-1})	400
	Sludge age (days)	15	Substrate loading (kg (m^3 d) $^{-1}$)	1.68
	Volumetric exchange (%)	70		
	Anaerobic/aerobic	0/1[3]		

[3] No anaerobic phase during the cycle time.

Period	Process Characteristics		Influent Characteristics	
6	Filling time (min.)	7	Carbon source	Caproic acid Glycerol
	Settling time (min.)	2		
	Total cycle time (min.)	360	COD (mg l^{-1})	450
	Sludge age (days)	15	Substrate loading (kg (m^3 d) $^{-1}$)	1.17
	Volumetric exchange (%)	65		
	Anaerobic/aerobic	0/1[3]		
7	Filling time (min.)	7	Carbon source	Caproic acid Glycerol
	Settling time (min.)	2		
	Total cycle time (min.)	360	COD (mg l^{-1})	900
	Sludge age (days)	15	Substrate loading (kg (m^3 d) $^{-1}$)	2.34
	Volumetric exchange (%)	65		
	Anaerobic/aerobic	0/1[3]		
8	Filling time (min.)	7	Carbon source	Caproic acid Glycerol
	Settling time (min.)	2		
	Total cycle time (min.)	360	COD (mg l^{-1})	1350
	Sludge age (days)	15	Substrate loading (kg (m^3 d) $^{-1}$)	3.51
	Volumetric exchange (%)	65		
	Anaerobic/aerobic	0/1[3]		
9	Filling	10	Carbon source	Glucose Peptone
	Settling time (min.)	2		
	Total cycle time (min.)	240	COD (mg l^{-1})	800
	Sludge age (days)	10	Substrate loading (kg (m^3 d) $^{-1}$)	2.24
	Volumetric exchange (%)	70		
	Anaerobic/aerobic	0/1[3]		
10	Filling time (min.)	10	Carbon source	Glucose Peptone
	Settling time (min.)	2		
	Total cycle time (min.)	360	COD (mg l^{-1})	400
	Sludge age (days)	10	Substrate loading (kg (m^3 d) $^{-1}$)	1.04
	Volumetric exchange (%)	65		
	Anaerobic/aerobic	3/7		
11	Filling time (min.)	10	Carbon source	Glucose Peptone
	Settling time (min.)	2		
	Total cycle time (min.)	480	COD (mg l^{-1})	400
	Sludge age (days)	10	Substrate loading (kg (m^3 d) $^{-1}$)	1.2
	Volumetric exchange (%)	75		
	Anaerobic/aerobic	3/7		
12	Filling time (min.)	10	Carbon source	Glucose Peptone
	Settling time (min.)	2		
	Total cycle time (min.)	360	COD (mg l^{-1})	200
	Sludge age (days)	10	Substrate loading (kg (m^3 d) $^{-1}$)	0.6
	Volumetric exchange (%)	75		
	Anaerobic/aerobic	3/7		
13	Filling time (min.)	12	Carbon source	Glucose Peptone
	Settling time (min.)	24		
	Total cycle time (min.)	360	COD (mg l^{-1})	800
	Sludge age (days)	30	Substrate loading (kg (m^3 d) $^{-1}$)	2.4
	Volumetric exchange (%)	75		
	Anaerobic/aerobic	3/7		

Period	Process Characteristics		Influent Characteristics	
14	Filling time (min.)	10	Carbon source	Glucose Peptone
	Settling time (min.)	10		
	Total cycle time (min.)	360	COD (mg l^{-1})	400
	Sludge age (days)	30	Substrate loading (kg (m^3 d) $^{-1}$)	1.2
	Volumetric exchange (%)	75		
	Anaerobic/aerobic	3/7		
15	Filling time (min.)	8	Carbon source	Glucose Peptone
	Settling time (min.)	2		
	Total cycle time (min.)	360	COD (mg l^{-1})	1200
	Sludge age (days)	10	Substrate loading (kg (m^3 d) $^{-1}$)	3.6
	Volumetric exchange (%)	70		
	Anaerobic/aerobic	3/7		

4.3 General Analysis and Chemicals

All chemicals were used in analytical quality, if not noted differently.

4.3.1 Ammonium, Nitrate, Nitrite and COD-Analysis

Photometric test kit, Dr. Lange.

4.3.2 Carbohydrate Determination

Carbohydrates were determined quantitatively according to the modified description of Dreywood (1946). 1 ml of the liquid sample or standard, 2 ml sulphuric acid (75 %) and 2 ml of fresh “anthron” solution (2 g/l in sulphuric acid, 75 %) were added and mixed immediately. The reaction took place within 15 min. while boiling the sample in a water bath. After cooling down to room temperature the absorption at a wave length of 578 nm was determined. As a standard, glucose in concentrations of 0 to 300 mg/l was used.

4.3.3 Filtration

Cellulosenitrate membrane filter, 0.45µm, Schleicher & Schüll.

4.3.4 Polyhydroxy Butyric Acid Determination

Polyhydroxybutyrate (PHB) is an important storage product in the metabolism of phosphate accumulating organisms (PAOs). PHB was analysed in a total of six granular sludge samples taken in one SBR cycle in steady state conditions of period 10 (Table 2) following a protocol modified from Brdjanovic *et al.* (1999). PHB was depolymerised with hydrochloric acid and converted into 3-hydroxy propylester. After extraction with water a solution in dichlorethane remained which was injected into a gas chromatograph.

Granular sludge samples were centrifuged at 3000 g and freeze-dried. PHB in freeze-dried biomass samples was hydrolysed by adding 1.5 ml of a solution of hydrochloric acid (32 %) in 1-propanol [1:4], 1.5 ml of dichlorethane and 0.5 ml of an internal standard solution containing 20 mg l^{-1} benzoic acid in 1-propanol. The mixture was heated in a tightly closed test tube for two hours at 100 °C and occasionally stirred. Water (3 ml) was added and the tube was centrifuged for complete separation of the two phases. 1 ml of the organic layer was dried over night by additing sodium sulphate. 1 µl of the clear solution was injected into a gas chromatograph (column: DB FFAP) at 200 °C. Detection temperature of the flame ionisator (FID) was 240 °C. Pure PHB (Fluka) was used for calibration.

4.3.5 Synthetic Wastewater

Carbon sources: caproic acid, Merck; glucose, Sigma; glycerol, Fluka; peptone, Sigma; sodiumacetate, Merck; d-sucrose, Merck.

Nutrients: ammoniumchloride, Sigma; ammoniumhydrogencarbonate, Sigma; calcium-chloride-hexahydrat, Merck; di-sodiumhydrogenphophate, Merck; iron(III)-chloride-hexahydrat, Merck; potassiumhydrogencarbonate, Merck; magnesiumsulfate-heptahydrat, Merck.

Micronutrients from Merck: $Al_2(SO_4)_3 \times 18\ H_2O$; $CoSO_4 \times 7\ H_2O$; $CuSO_4 \times 5\ H_2O$; $K_2Cr_2O_7 \times 7\ H_2O$; KJ; $MnSO_4 \times 7\ H_2O$; $Na_2B_4O_7 \times 10\ H_2O$; $Na_2MoO_4 \times 2\ H_2O$; $NiSO_4 \times 7\ H_2O$; $ZnSO_4 \times 7\ H_2O$.

4.3.6 EPS-Extraction

EPS was extracted according to the previously described method (Frølund *et al.*, 1996) using an ion exchange resin DOWEX 50 x 8 (Fluka) and additional chemicals: potassiumchlorid, Merck; sodiumchloride, Merck; sodiumdihydrogenphophate-dihydrate, Merck; trisodiumphosphate-dodecahydrate, Merck.

4.4 Microsensor Measurement

4.4.1 Micro-Optodes

To investigate the micro-environment *in situ,* different microsensors were used and micro-profiles were determined within single granula, harvested from the reactor. Microsensors rely on either electrochemical or optical principles (Kühl and Revsbech, 2000). For this study, oxygen was measured by micro-optodes which are fiber-optical microsensors (Klimant *et al.*, 1995). The optode system Microx I was manufactured by Presens Precision Sensing GmbH, Neuburg, Germany. The sensors are characterised by a measuring range from 0-800 % oxygen, accuracy of 1 % in air and a typical tip diameter of 30-40 µm. Compared to other

electrochemical sensors no oxygen is consumed during measurement and no approaching flow is necessary. Temporal resolution is smaller than 1 sec and spatial resolution is smaller than 50 µm. Optodes can be stored for months. The micro-optodes were calibrated right before the experiment at room temperature using a two-point calibration.

4.4.2 LIX Microsensors

Additional electrochemical micro-sensors with an ion-selective membrane were used. These LIX (Liquid Ion Exchanger) sensors can be made very small (1µm), but often suffer from a short lifetime (24 h) and a low selectivity of the membrane (Santegoeds *et al.*, 1998). LIX sensors for NH_4^+ (de Beer and van den Heuvel, 1988), NO_2^- (de Beer *et al.*, 1997) and NO_3^- (de Beer and Sweerts, 1989) with solidified tips and protein coatings were manufactured as described previously (de Beer *et al.*, 1997b). The tip diameters were 6-8 µm for NH_4^+ and NO_3^- microsensors and 13-16 µm for NO_2^- microsensors. Calibration was done in a dilution series of NH_4^+, NO_2^- and NO_3^- in the medium which was used for the measurements.

4.4.3 Experimental Set-Up

To measure chemical gradients in activated sludge flocs a vertical net-jet flow system was developed by Ploug *et al.* (1998). According to their technical description a similar flow cell was made from two plexiglas tubes with a nylon stocking in between (Figure 6 and b). Upward flow was applied that keeps the aggregates floating just above the netting. Aggregates were placed in the flow cell and perfused with medium. Temperature was kept constant to 20 ± 1 °C and pH remained constant (pH 7.3). After 15 min. of incubation micro-profiles were recorded by moving the sensor with a motor-driven micro-manipulator at depth steps of 25 µm from the bulk liquid into the aggregate. The position relative to the aggregate surface was determined with a dissection microscope (3M Wild, Leica).

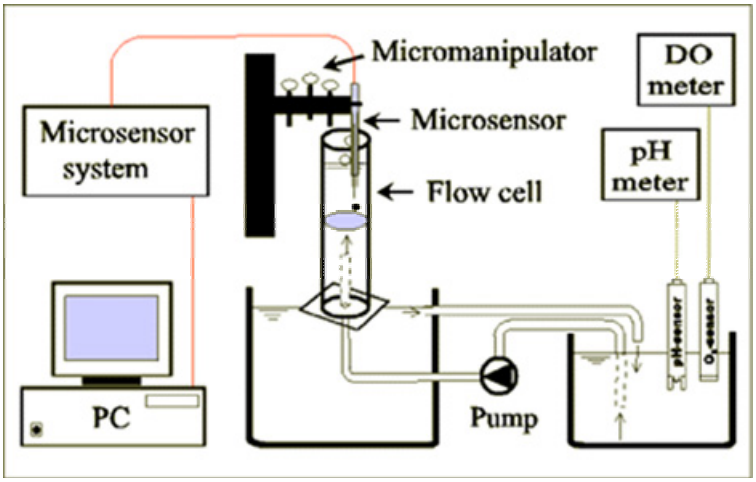


Figure 6a. Schematic View of the Micro-Sensor Set-Up

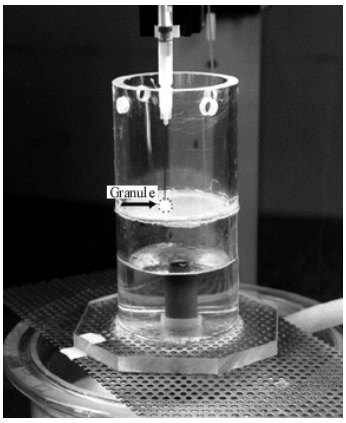


Figure 6b.
Micro-Sensor Flow Cell

4.5 Molecularbiological Methods

4.5.1 Granular Sludge Sampling and Cryo-Sectioning

In co-operation with the Lehrstuhl für Mikrobiologie, Technische Universität München, microbial population analyses were performed on granule samples taken 10 weeks after the start-up of the SBR reactor (section 4.2, Table 2, period 10). Samples of aerobic granular sludge were carefully removed from the SBR laboratory preserving their structural integrity. Subsequently, the granules were embedded in Cryo-embedding compound for low and medium temperatures (MICROM International GmbH, Walldorf, Germany). After freezing the samples to –40°C, the granules were sliced with the Cryotom MICROM HM500 (MICROM International GmbH, Walldorf, Germany) in 15 µm sections, which were immediately transferred to poly-L-lysine covered glass slides. Samples on slides were then fixed with 4 % (w/v) paraformaldehyde or ethanol (Amann, 1995) and stored for 4 h at 4°C. Afterwards, the slides were washed with phosphate buffered solution (PBS) (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2), transferred into 50 % (v/v) PBS/Ethanol and stored at –20 °C until use. Several other unfixed granules were stored at –20 °C for subsequent nucleic acid extraction (Etterer *et al.*, in preparation).

4.5.2 DNA Extraction from Aerobic Granular Sludge

Total genomic DNA of aerobic granular sludge was isolated with the Fastprep Bead-beater (BIO 101 Systems, Vista) and FastDNA kit (BIO 101 Systems, Vista) according to the standard protocol provided with the instrument.

4.5.3 PCR Amplification of 16S rDNA

To obtain almost full length 16S rDNA PCR-products, the aerobic granular sludge derived DNA was amplified with the forward primer 616V (*E.coli* positions 8-27; 5'-AGAGTTTGATYMTGGCTCAG-3') and the reverse primer 630R (*E.coli* positions 1529-1545: 5'-CAKAAAGGAGGTGATCC-3'). PCR was performed in a 96 micro well plate with a gradient cycler (Eppendorf, Hamburg, Germany). Reaction mixtures were prepared in a total volume of 50 µl containing 2 mM $MgCl_2$, 10 nmol of each deoxynucleoside triphosphate, 15 pmol of each primer, 100 ng of template DNA and 1.5 U of Taq DNA polymerase (Promega, Madison). Thermal cycling was carried out with an initial denaturation of 3 min. at 94 °C, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 52 °C for 50 s, and elongation at 72°C for 3 min. Cycling was completed by a final elongation step at 72°C for 10 min. Negative controls (no DNA added) were included in all sets of amplifications. The presence and size of amplification products were determined by agarose (0.8 %) gel electrophoresis of 5 µl aliquots of the PCR products.

4.5.4 Cloning and Sequencing

The granular sludge-derived 16S rDNA products were cloned directly by using the TOPO TA Cloning kit following the instructions of the manufacturer (Invitrogen, Groningen, The Netherlands). Plasmid-DNA was isolated with the Quiaprep spin miniprep kit (Quiagen, Hilden, Germany). Plasmids with an insert of the expected size were identified by agaraose (1.0 %) gel electrophoresis after EcoRI digestion (5 U, Eco RI-buffer for 3 h at 37 °C). Sequencing was done non-radioactively by using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit according to the instructions of the manufacturer (Amersham, Freiburg, Germany). The reaction mixtures were analyzed with an infrared automated DNA sequencer (model LiCor Longreadir DNA 4200, MWG - Biotech, Ebersberg, Germany). The complete sequences of the 16S rDNA fragments were determined by using M13 forward and reverse primers targeting vector sequences adjacent to the multiple cloning site.

4.5.5 Phylogenetic Analysis

The 16S rDNA retrieved in this study were added to the 16S rDNA data base of the Technische Universität München, Lehrstuhl für Mikrobiologie (currently encompassing more than 16.000 small subunit rRNA sequences), by using the ARB program package (Strunk and Ludwig, 1997). 16S rRNA sequences were aligned automatically using the respective tool of the ARB package. Subsequently, the alignments were corrected by visual inspection. Phylogenetic analysis of 16S rRNA sequences were performed by applying neighbor-joining, parsimony and maximum likelihood analysis (fast DNAml, Maidak *et al.*, 1996) to different data sets. Bootstrapping was performed using the PHYLIP parsimony tool (100x resampling) (Phylogeny Inference Package Version 3.5c, University of Washington, Seattle). Checks for

chimeric sequences were conducted by independently subjecting the first 5' 454 base positions, the middle 455 base positions, or the last 454 3' base positions for phylogenetic analysis (Etterer *et al.*, in preparation).

4.5.6 Probe Design, Fluorescence *in situ* Hybridisation, Microscopy, and Quantification

For the probes used in this study, sequences, target sites and optimal formamide concentrations in the hybridisation buffers are displayed in Table 3. Probe S-*-Clta-0467-a-A-18 (clta: clones affiliated to *Tolumonas auensis*) specific for the retrieved *Tolumonas auensis*-affiliated sequences was designed using the probe design tool of the ARB package. Probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives from Thermohybaid Interactiva division (Ulm, Germany). Hybridisations were performed as described by Amann (1995). Simultaneous hybridisation with probes requiring different stringency was realised by a successive-hybridisation procedure (Wagner *et al.*, 1994c). Optimal hybridisation conditions for probe S-*-Clta-0467-a-A-18 were determined by using the hybridisation and wash buffers described by Manz *et al.* (1992). An *in situ* probe dissociation curve was recorded by measuring the relative fluorescence intensity of granular sludge bacteria after hybridisation with probe S-*-Clta-0467-a-A-18 at different stringencies as described by Daims *et al.* (1999).

Dual staining of cells hybridised with Cy3-labeled probes with SYBR Green I (FMC Bioproducts, Rockland) was performed as previously described at Schmid *et al.* (2000). Slides were washed briefly with ddH_2O, air-dried and embedded in Citifluor (Citifluor Ltd., Canterbury, UK). For image acquisitions a Zeiss LSM 510 scanning confocal microscope (Zeiss, Jena, Germany) equipped with a UV laser (351 and 364nm), an Ar ion laser (458 and 488 nm) and two HeNe lasers (543 and 633nm) was used together with the standard software package delivered with the instrument (version 2.1). The EUB/SYBR Green I ratio was determined by using an equimolar mixture of the probes EUB338, EUB338-II, and EUB338-III (30 ng of each probe labeled with Cy3; Daims *et al.,* 1999) by applying the digital image analysis procedure described below. For quantification of granular sludge bacteria related to the specific subpopulations, subclass and species specific Cy3 labelled probes were used together with the Cy5 labeled bacterial probe set (EUB338, EUB338-II, EUB338-III) for simultaneous hybridisation. The ratio of the area of cells stained with SYBR Green I or the specific probe, respectively, vs. the ratio of the area of those cells labeled with the bacterial probes was determined as described at Schmid *et al.* (2000) and Daims *et al.* (2001).

Table 3. Oligonucleotide Probe Sequences, Target Sites, Formamide Concentrations in the Hybridisation Buffer Required for Specific *In Situ* Hybridisation

Trivial name (reference)	OPD[a] designation	Specificity	Sequence 5'-3'	% Formamide/ mM [NaCl]	Target site[b]
ACA 23a (Wagner *et al.,* 1994b)	S-P-Acin-0652-a-A-18	*Acinetobacteria sp.*	ATCCTCTCCCATACTCTA	35/80	16S rRNA, 652-669
Act 1458 (Rabus *et al.,* 1999)	S-P-AzTh-0338-a-A-21	*Azoarcus/Thauera*	GAATCTCACCGTGGTAAGCGC	50/28	16S rRNA, 1458-1479
Alf968 (Neef ,1997)	S-P-Alf-0968-a-A-18	*Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	20/225	16S, rRNA 968-985
Bet42a (Manz *et al.,* 1992)	L-P-Bet-1027-a-A-17	*Betaproteobacteria*	GCCTTCCCACTTCGTTT	35/80	23S rRNA, 1027-1043
CF319a (Manz *et al.*, 1992)	S-P-CF-0319-a-A-18	*Cytophaga/Flavobacterium*	TGGTCCGTGTCTCAGTAC	35/80	16S rRNA, 319-336
Eub338 (Amann *et al.,* 1990)	S-D-Bact-0338-a-A-18	*Bacteria*	GCTGCCTCCCGTAGGAGT	0/900	16S rRNA, 338-355
Eub338II (Daims *et al.,* 1999)	S-D-Bact-0338-a-A-18	Bacterial lineages not covered by probe EUB338 and EUB 338 III	GCAGCCACCCGTAGGTGT	0/900	16S rRNA, 338-355

Trivial name (reference)	OPD[a] designation	Specificity	Sequence 5'-3'	% Formamide/ mM [NaCl]	Target site[b]
Euk 516 (Amann *et al.*,1995)	S-D-Euk-0516-a-A-16	*Eucarya*	ACCAGACTTGCCCTCC	0/900	18S rRNA, 516-531
Gam42a (Manz *et al.*, 1992)	L-P-Gam-1027-a-A-17	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	35/80	23S rRNA, 1027-1043
Hgc69a (Roller *et al.,* 1994)	L-P-HGC-1901-a-A-18	*Actinobacteria*	TATAGTTACCACCGCCGT	25/159	23S rRNA, 1901-1918
Nso1225 (Mobarry *et al.,* 1996)	S-P-Betao-1225-a-A-20	Ammonia oxidizers of the *Betaproteobacteria*	CGCCATTGTATTACGTGTGA	35/80	16S rRNA, 1225-1244
Nso 190 (Mobarry *et al.*, 1996)	S-P-Betao-0190-a-A-19	Ammonia oxidizers of the *Betaproteobacteria*	CGATCCCCTGCTTTTCTCC	55/20	16S rRNA, 190-208
Ldi 23a (Wagner *et al.,* 1994a; c)	S-S-Ldi-0649-a-A-18	*Leptothrix discophora* and relatives	CTCTGCCGCACTCCAGCT	35/80	16S rRNA, 649-666
Lgcbc[c)] (Meier *et al.*, 1999)	S-P-LGC-0354-a-A-18	Some *Firmicutes*	TGGAAGATTCCCTACTGC	20/225	16S rRNA, 354-371
Lgcb[c)] (Meier *et al.*, 1999)	S-P-LGC-0354-b-A-18	Some *Firmicutes*	CGGAAGATTCCCTACTGC	20/225	16S rRNA, 354-371
Lgcc[c)] (Meier *et al.*, 1999)	S-P-LGC-0354-c-A-18	Some *Firmicutes*	CCGAAGATTCCCTACTGC	20/225	16S rRNA, 354-371

Trivial name (reference)	OPD[a] designation	Specificity	Sequence 5'-3'	% Formamide/ mM [NaCl]	Target site[b]
PAO651 (Crocetti *et al.*, 2000)	S-*-PAO-0651-a-A-18	Phosphate accumulating bacteria affiliated to *Rodocyclus*	CCCTCTGCCAAACTCCAG	35/80	16S rRNA, 651-668
Pla46 (Neef *et al.,* 1998)	S-P-Planc-0046-a-A-18	*Planctomycetales*	GACTTGCATGCCTAATCC	25/159	16S rRNA, 46-63
Sna 23a (Wagner *et al.,* 1994a; c)	S-S-Sna-0656-a-A-18	*Sphaerotilus natans* and relatives	CATCCCCCTCTACCGTAC	45/40	16S rRNA, 656-673
Zra 23a (Rosello-Mora *et al.,* 1995)	S-S-Zora-0647-a-A-18	*Zoogloea ramigera*^{T}	CTGCCGTACTCTAGTTAT	35/80	16S rRNA, 647-664
- (Juretschko *et al.*, 2002)	S-*-Zora-1414-a-A-20	*Zoogloea ramigera*^{T} and affiliated molecular isolates	TTCTGGTAAACCCCACTCCC	25/159	16S rRNA, 1414-1433
- (Etterer *et al.*, in preparation)	S-*-Clta-0467-a-A-18	Aerobic granules derived clones affiliated to *Tolumonas auensis*	ACGTCAATGTTGATGCGT	15/318	16S rRNA, 467-484

[a)]Oligonucleotide probe database (Alm *et al.*, 1996), [b)] rRNA positions, E. coli numbering (Brosius *et al.*, 1981),

[c)]Probes Lgca, Lgcb and Lgcc were applied together

4.6 Physico-chemical Parameters

4.6.1 Porosity Estimation

In general, the force balance for a floc or a granula moving steadily in an infinite medium can be described as follows (Li, 1992; Huang, 1993):

$$1 - e = \frac{3\,\rho_w\,\Omega\,C_D}{4\,g\,(\rho_p - \rho_w)\,d_g}\,v^2 \qquad (1)$$

with

e: granula porosity

Ω: Factor that describes the ratio of the resistance experienced by a granule to that of an equivalent solid sphere

ρ_w: Density of water

ρ_p: Density of the primary particles comprising the floc

C_D: Drag coefficient

Using the free-settling test, only terminal settling velocity and aggregate diameter are available. Hence, four unknowns (ρ_p, Ω, C_D and e) in the equation (1) exist. To estimate aggregate porosity the parameters ρ_p, Ω and C_D are required. ρ_p is the density of the primary particles (section 2.2.4) comprising the floc. It is not equal with dried solid density, which can only serve as an upper limit. According to Lee *et al.* (1996) equation (2) is used to estimate ρ_p:

$$\rho_p = \frac{1 + W_a}{1/\rho_s + W_a/\rho_w} \qquad (2)$$

where W_a is kg bound water / kg dried solid. Factor Ω, which describes the ratio of the resistance experienced by a granule to that of an equivalent solid sphere, can be calculated for a highly porous sphere as follows (Neale *et al.*, 1973):

$$\Omega = \frac{2\beta^2 * [1 - (\tanh\beta)/\beta]}{2\beta^2 + 3 * [1 - (\tanh\beta)/\beta]} \qquad (3)$$

where

$$\beta = \frac{d_g}{2\sqrt{k}}$$

is a dimensionless floc diameter and k is the floc permeability (m^2). Floc permeability evaluation is usually based on permeability models. A review over some typical models which are generally accepted in literature can be found in Lee *et al.* (1996). Lee and coworkers (1996) also described the model provided by Brinkman, which is the only one used for this calculations. In all models, the two unknowns porosity and diameter of the primary particles are necessary to estimate floc permeability. Hence, assumptions were made for the calculations. Primary particle diameters range from 1 to 20 µm (Lee *et al.,* 1996). Drag coefficient C_D is a function of the Reynolds number and the aggregate sphericity (Namer, 1993). The drag coefficient for granules was estimated according to Ganser (1993).

4.6.2 Determination of Settling Velocity and Size of Aerobic Granula

Final settling velocity of single granula harvested from the reactor was measured by applying free-settling tests according to Lee *et al.* (1996). A plastic cylinder (6 cm in diameter and 90 cm in height) filled with the clear liquid phase of the reactor was used for the test. Single granules were put into the cylinder and could reach their final settling velocity in the upper 30 cm of the water column. Then the settling time for the distance of 50 cm was taken manually with an accuracy of ± 0.5 s. Temperature was 20 ± 1 °C. All settling tests were performed twice and the average was determined. After each settling test aerobic granules were directly photographed with a digital zoom camera, Kodak DC 260. Using this pictures the average granula diameter could be determined by digital image analysis.

4.6.3 Measurement of Granula Density

For the determination of aggregate density several methods are available, among others sedimentation techniques (Li, 1986). Some complications with sedimentation methods exist, mainly because of the hydrodynamic behaviour of aggregates. Since solid particles are usually not spherical, expressions for drag coefficient of spheres need to be modified. Another uncertainty arises from the porosity and the possibility of flow through the floc structure (Li, 1988; Gregory, 1997). Only a few reports indicate that the density of flocs could be measured directly (Lagvankar, 1968). Isopycnic density gradient centrifugation was used to determine granula density. Dammel (1991) checked the method and found it reliable and reproducible for activated sludge solids. The method is commonly used for separation of cells and subcellular particles. During centrifugation a fluid column is constructed whose density increases continuously with depth. Particles will settle to a level in the column where their density equals that of the surrounding fluid. The density gradient medium is a colloid suspension[4] of heterogeneously distributed silica particles. Characteristics of the suspension as noted by Laurent *et al.*, 1980 are:

- The density gradient can be accurately calibrated with respect to density versus depth.
- An appropriate density range for activated sludge solids, 1.01-1.10 g/ml is available.

[4] Percoll ®, Pharmacia Biotech AB, Uppsala Sweden.

- Reported densities for bacterial cells range from 1.04 to 1.1 g/ml (Woldringh *et al.*, 1981; Scherer, 1983).
- The suspension is non-toxic and during the test flocs will remain viable with limited cell lysis.

The centrifugation medium was prepared by diluting the commercial suspension with a 1.5 M NaCl stock solution to a final ionic strength of 0.15 M NaCl. Ionic strength of the medium is important since the buoyant density of the biological material is affected by varying the ionic strength of the centrifugation medium (Vincent, 1984; Dammel, 1991).

Calibration and Measurement

For the calibration of the diluted centrifugation medium, density marker beads with a characteristic density were used. Beads of the same density form a band which settles to a depth in the gradient at matching density. Plotting the depth from the surface of the gradient to each band against the density of each band establishes the depth versus density curve (Pharmacia, 1986). One test tube containing the density marker beads was always centrifuged together with the samples to be measured. Temperature (20 °C), time and centrifugation rates were kept constant. 10 ml of the diluted colloidal solution was placed into each of six identical test tubes and centrifuged for 120 min. at 12,000 rpm to establish the gradients. The depth of each band of density marker bead was measured to the nearest 0.5 mm. Then depth versus density was plotted. The density versus distance relationship between each band was assumed to be linear. The depth of solids in the gradient was measured, and the solids' density was determined from density versus depth graph.

4.6.4 Adsorption Test

The surface area of porous solids can be determined by dye adsorption techniques. Lissamine Scarlet 4R, colour index generic name: Acid Red 18 (AR18), has been reported to be suitable for dye adsorption measurement on activated sludge and biofilm (Smith and Coackley, 1983, 1984; Zhang and Bishop, 1994, a; b). AR 18 (C.I. number 16255) has a molecular weight of 604.48 and a molecular area flat of 196 Å.

Activated sludge samples were taken at the municipal treatment plant of the city of Garching. Granular sludge samples were taken in period 10 (Table 2). Dye adsorption tests were carried out in 100 ml flasks at room temperature (21 ± 1 °C). Each flask contained 20-30 ml of dye test solution, with a known initial dye concentration of 20 mg/l. Activated sludge suspension (20 ml) was added and acidified with 5 M HCl to a pH 2.5. Then the flasks were shaken for 1 hour (shaking velocity: 140 $min.^{-1}$) to establish equilibrium conditions for complete adsorption. Afterwards, the mixtures were centrifuged at 5000 rpm for 10 min. Then the supernatant was collected and diluted, if necessary (up to 1:20), with DI water before spectrophotometric measurements. The concentrations of dye remaining in solutions at

equilibrium were measured on a UV-1601 SHIMADZU uv-visible Spectrophotometer, in the glass micro-cuvettes (1 cm light path) at optimum wavelength 505 nm.

4.6.5 Shear Sensitivity Test

Break-up experiments were performed in order to investigate the effect of solid content and turbulent shear intensity on the degree of granular sludge dispersion. Activated sludge samples were taken at the municipal treatment plant of the city of Garching. Granular sludge samples were collected from the laboratory SBR, period 10 (Table 2). In all tests 1000 ml of sludge was placed in a baffled reaction chamber consisting of a 105 mm diameter cylinder with four 11 mm wide vertical baffles. All tests were performed at room temperature (20 ± 1°C). A single-bladed paddle of width 5.0 cm and height 1.2 cm was placed centrally in the horizontal plane, 4 cm above the bottom of the cylinder, as measured from the centre of the paddle in the vertical plane. The paddle speed was provided by a Heidolph electronic mixer and controlled from tachometer measurements.

An identical set-up as described by Mikkelsen and Keiding (1999) was used. Hence paddle shaft torque measurements were not executed, but taken from Mikkelsen (1999). The applied shear was 800 s^{-1}. Related impeller Reynolds number was always > 1000, which normally corresponds to turbulent conditions. The corresponding Kolmogoroff micro scales of turbulence was η = 40 µm. According to Parker *et al.* (1970), activated sludge primary particles sizes range from 0.5-5 µm. Hence, primary particles were smaller than the microscale, which is a requirement for the use of G for turbulence characterisation (Cleasby, 1984). This was confirmed by shear experiments with different paddles (Mikkelsen, 1999). Paddle configuration had no effect using the same shear (G = 800 s^{-1}).

To test the effect of solid content, sludge was diluted with supernatant to resulting solids concentration of 1.8, 2.4 and 3.1 g SS l^{-1}. Sludge samples (10 ml) for turbidity measurements were sampled in 20 min. intervals from 0 to 300 min. of shearing time. Supernatant turbidity was measured after 2 min. centrifugation at 3000 $min.^{-1}$. Turbidity was determined with a Hach turbidimeter (type 2100). To ensure that the measured turbidities were descriptors of primary particle numbers and not affected by small aggregates, tests of sonication were performed. Turbidities did not vary after sonication. Hence, the turbidities were assumed to describe the number of primary particles in a linear relationship.

4.7 Respirometric Measurements

At the end of the cycle 1000 ml granular sludge were taken from the sequencing batch reactor. The sludge sample was settled and the supernatant was then replaced with tap water in order to reduce the concentration of biodegradable COD. In order to avoid any nutrient limitation the following nutrients were added to the sludge: $MgSO_4$: 160 mg l^{-1}, FeCl3: 2 mg l^{-1}, $CaCl_2$: 410 mg l^{-1}, NH_4Cl-N: 6.5 mg l^{-1} and phosphate buffer (pH 7.3). To inhibit nitrification, N-allylthiourea (ATU) was added to a concentration of 13 mg l^{-1}. The sludge was then aerated for at least five hours at room temperature before it was used for experiments.

Respiration was measured, following the "Deutsche Einheitsverfahren" (DIN 38414, T6, S6), in an experimental set-up consisting of a closed measuring batch reactor (volume 330 ml). The system was kept at room temperature of 20 ± 0.5 °C. Oxygen was measured by means of an oxygen electrode (Oxi 196, WTW). Organic substrate (mixture of: acetate, ethanol, peptone, meat extract, glucose, ; 10 ml) was added, oxygen uptake rate (OUR) measurements were repeated three times and the average was determined.

Since the activity of nitrifying bacteria is considerably influenced by the pH value, the pH value was measured before and after the batch test. Representative samples were taken from the laboratory SBR. Before the OUR determination the sample was aerated for 5 h. The ammonium level to determine the maximum autotrophic oxygen uptake rate $OUR_{A,max}$ has to be sufficiently high but low enough in order to avoid free ammonia inhibition. According to this an ammonia level of 15 mg NH_4-N l^{-1} is generally sufficient for activated sludge (Nowak *et al.*, 1994). To inhibit nitrification a concentration of 13 mg l^{-1} ATU was added. To guarantee a complete inhibition a reaction time of 30 min. was chosen.

4.8 Technical Equipment

Centrifugation Varifuge 3.2 RS, Heraeus; Centrifuge 4517R, Eppendorf.

COD Digital photometer system ISIS 6000, Dr. Bruno Lange

DOC Total Carbon Monitor 480, Carl Erba Instruments

Dry freezer VaCo I, Zirbus

Gaschromatograph 5890 Series II, Hewlett-Packard, column: HP-5, Ultra 2, 50 m

Mass detector 5871 Series II, Hewlett-Packard

Turbidimeter Type 2100, Hach

5 RESULTS

5.1 Formation of Aerobic Granular Sludge

5.1.1 General Observations

The reactors were operated under different process conditions (section 4.2, Table 2), but generally the filling phase was kept short (7-10 min.). The substrate solution was pumped into the reactor 2-3 min. before tab water was added for dilution. After the static fill the reactor was aerated for one minute to mix the concentrated substrate.

Static fill was typical for all different process conditions. Aeration, which provided the only mixing in the reactor system, was realised by compressed air pumped through porous stones. The porous stones were controlled weekly and changed if aeration was hampered. Shearing effects in the reactor were related mainly to the air flow rate, however, the described air bubble modification might have influenced the shearing process as well. The production of the extracellular slime and partly the blocking of the aeration system occurred not regularly, but a correlation to the granulation process could not be recognised.

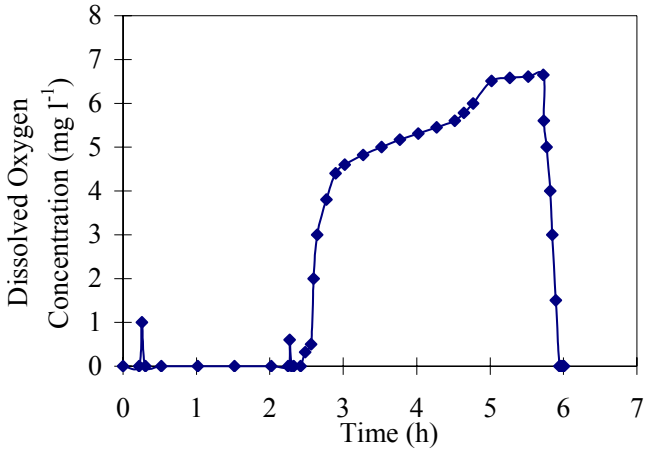


Figure 7. Typical DO Profile during Steady State Cycle of the SBR (Period 10,Table 2)

The time course of the DO concentration in Figure 7 is characteristic for an SBR cycle in steady state conditions (section 4.2, Table 2, period 10). Due to an anaerobic phase at the beginning of the cycle the aeration in the reactor system started nearly 100 min. after filling. Within 30 to 40 min. the DO level reached to concentrations above 4-4.5 mg O_2 l^{-1} and slowly increased to 6 mg O_2 l^{-1} during hours 3 to 5 in the cycle. The COD level decreased simultaneously after 5/6 of the total time cycle to 15-20 % of the influent concentration.

For the first two weeks after the inoculation flocculent activated sludge typically dominated in the experimental reactors. Depending on the environmental conditions (carbon source, aeration) and the process conditions (hydraulic retention time, volumetric exchange ratio,

settling time) first granules appeared in the second or third week after start-up. Typically for the fourth and fifth week was that granular and flocculent sludge were simultaneously present in the reactor. The first days after inoculation selective pressure was stepwise increased, hence the VER in the different experimental periods 4 to 15 (section 4.2, Table 2.) was increased from 50 % up to 75 %.

5.1.2 Significance of Different Carbon Sources

The influence of different environmental conditions on the granulation was investigated by means of synthetic wastewater which was used under various process conditions (Table 2, period 2-15). The relative nitrogen and phosphorus content in the different substrate compositions was kept constant within values of 100:4.5:1 and 100:6:2 (COD:N:P).

A total of six different carbon sources in four different combinations (Table 4) were studied. Granulation could not be achieved with all carbon sources tested in this study. First tests with acetate as single carbon source showed that granules appeared in the SBR after 20 days of operation, but activated sludge flocs could not be repressed. A mixture of different low molecular and easy degradable carbon sources (caproic acid, glycerol, sucrose) was chosen for further investigations. Experiments were stopped after 4-5 weeks if no granules were formed or if filamentous organisms dominated the granular structure, which was controlled by microscopic observations (Table 2 and 4, period 3, 4).

Sludge structure of the resulting granular sludge in the different periods varied significantly. In period 3 and 4 the growing sludge flocs had good settling properties and could not be effectively washed out of the reactor. Probably sucrose promoted the production of slimy extracellular polymeric substances in period 3 and 4. Increasing the VER from 50 to 60 % did not influence the selection process significantly. Hence, in the following experiments sucrose was omitted in periods 5 to 8.

By using caproic acid and glycerol as carbon sources granulation could be enhanced. After 15 to 20 days granules were observed in the reactors. In period 6 granules could not be formed within 28 days. It is assumed that the result was not related to the carbon source, since for period 5, 7 and 8 granules could be cultivated. The answer why granulation failed in period 6 might be found in variations of the microbial composition of the activated sludge used for inoculation.

Results of the periods 5 to 8 were satisfying regarding the granulation process, however, the experiments of Zhu et al. (2001) and Zhu and Wilderer (2003) showed that granules with a more compact structure can be formed although very similar process conditions were used. Zhu *et al.* (2001) used glucose and peptone as carbon sources. A mixture of glucose and peptone was used in the final experimental periods (Table 2 and 4, period 9-15). These carbon sources showed to be a good basis for granulation process.

Table 4. Investigated Carbon Sources and Observed Effects regarding Granulation

Carbon Source	Period	Observed Effects
Acetate	2	Filamentous granules after 20 days; fibrous surface
Caproic acid + Glycerol + Sucrose	3	Filamentous and slimy flocs; no granules after 28 days; Intensive biofilm growth at the reactor wall
	4	Filamentous and slimy flocs; some filamentous granules after 25 days
Caproic acid + Glycerol	5	Filamentous granules after 20 days; flocs dominating
	6	Filamentous flocs, no granules after 28 days
	7	Filamentous granules after 20 days; fibrous surface
	8	Filamentous granules after 15 days; fibrous surface
Glucose + Peptone	9	Filamentous granules after 30 days, smooth surface
	10	Filamentous, but compact granules after 12 days, smooth surface
	11	Filamentous, but compact granules after 22 days, smooth surface
	12	Filamentous, but compact granules after 24 days, smooth surface
	13	Filamentous, but compact granules after 28 days, smooth surface
	14	Filamentous, but compact granules after 20 days, smooth surface
	15	Filamentous, but compact granules after 20 days, smooth surface

Summarising the effects of different carbon sources, it has to be stated that the granulation process was possible with nearly all tested low molecular and readily biodegradable substrates. Since different process conditions (e.g. total cycle time, VER) were applied and the granulation process lasted for 2-4 weeks, conclusions about the effects of different carbon sources are limited. In period 5 to 8, without using sucrose the production of voluminous EPS could be avoided and an improved granulation compared to period 4 could be achieved. How the microbial structure was influenced by the carbon source was not examined in detail but light-microscopic investigations (section 5.2) showed that filamentous organisms were initially present in all granules of the different periods.

5.1.3 Significance of Different Process Characteristics

Since the settling time was kept short during the start-up period a wash-out of non-readily settleable biomass took place. To characterise the influence of various process conditions (e.g. substrate loading, SRT etc.) two SBR were run in parallel. The technical reactor conditions and influent characteristics for both reactors were identical.

In experimental periods 6 to 8 different substrate loading conditions were compared. Granulation could not be observed within four weeks after inoculation in period 6 while in

period 7 and 8 granules were formed after 20 and 15 days, respectively. The influent COD concentration in period 6 was 450 mg O_2 l^{-1} compared to a much higher influent concentration in period 7 (influent COD 900 mg O_2 l^{-1}) and 8 (influent COD 1350 mg O_2 l^{-1}). Additionally to the experiments with caproic acid and glycerol, granules were cultivated under similar loading conditions but with glucose and peptone as carbon sources. Even an influent COD concentration of 200 mg O_2 l^{-1} (section 4.2, Table 2, period 12) could be successfully used to grow granular sludge.

A solids retention time (SRT) in the range between 10 and 30 days had no direct effect on the formation of aerobic granules. The introduction of an anaerobic phase at the beginning of the cycle resulted in a strengthening of the granular structure. Compared to periods 5 to 8 (Figure 9) the granular surface in periods 10 to 14 (Figure 8) was smoother and structured more compactly. The granulation was observed for both, aerobic and partly anaerobic/aerobic process conditions. The total cycle duration was varied between four and eight hours and different loading and substrate composition were applied. Granulation seemed not to be influenced by this parameter. Granules were cultivated in period 5, 10 and 11 with total cycle length of 4, 6 and 8 hours, respectively.

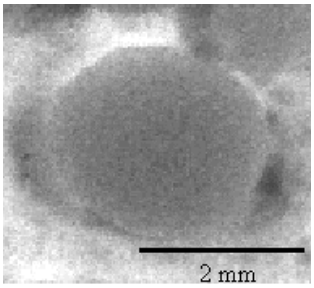


Figure 8. Typical Brown Granula of Period 10 with Smooth Surface

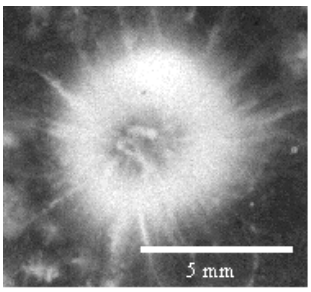


Figure 9. Typical White Granula of Period 5 with Filamentous Surface

Additionally in period 10 the effect of increased Ca^{2+}-ion concentrations on the granulation was investigated. The average influent concentration of 45 to 50 mg Ca^{2+}/l, which was close to tab water concentration, was increased up to 85 mg Ca^{2+}/l by adding calcium salts. Two SBR were inoculated and run in parallel for six weeks. During the time no enhancement of the granulation process, regarding a faster development of granules, could be observed.

5.1.4 Biomass Content and Settling Properties

Generally, for all periods with a successful granulation first small filamentous granules of about 300-400 µm diameter appeared after 12 to 15 days after reactor start-up. However, enhanced granulation took place between the 20. and 30. day. In the first weeks flocs still remained dominant. By the time granules accumulated and three to four weeks after inoculation, the biomass in the reactor mainly consisted of aerobic granules. Possibly due to

shearing effects by the intense aeration, granules became spherical with a smooth surface. Up to third and fourth week after the first appearance the average granula diameter in the different experimental periods typically increased. Hence, steady state conditions were reached after six to eight weeks after inoculation. Light microscopic observations (section 5.2.1) indicated that filamentous organisms were present and were involved in the overall structure of the aggregates, but did not grow away from the granules into the bulk liquid.

Table 5. Diameter, Biomass Content and Settling Properties of Aerobic Granular Sludge in different Experimental Periods

Process Characteristics				**Properties of the Granular Sludge**			
Period Number (referring to Table 2)	Influent COD ($mg\ l^{-1}$)	Substrate Loading ($kg\ (m^3\ d)^{-1}$)	Cycle Duration (h)	Steady State Diameter * (mm)	Biomass Content ($g\ SS\ l^{-1}$)	Settling Velocity * ($m\ h^{-1}$)	SVI ($ml\ g^{-1}$)
2	1200	3.6	4	1.5-4.9	6-8	35	70
8	1350	3.51	6	3.0-10.0	7-8	40	80
9	800	2.24	4	1.4-4.8	5-6	36	50
10	400	1.04	6	0.5-2.0	10-13	30	20
11	400	1.2	8	0.2-1.5	3-6	10	60
12	200	0.6	6	0.5-1.5	8-11	15	30
13	800	2.4	6	0.5-2.0	8-11	20	40
14	400	1.2	6	0.4-2.0	5-7	-	-
15	1200	3.6	6	1.1-6.5	6-7	39	60

* Results of 80 single granules harvested from the reactor were determined.

Comparing the different granular sludges formed in the experimental reactors, several differences are obvious (Table 5). Increased organic loading could directly affect the diameter of the granular sludge. For loading conditions of more than 2.4 $kg\ (m^3\ d)^{-1}$ the aggregate diameter ranged from 1.1 to 10 mm, whereas for lower loading the diameter was 2.0 mm or smaller. High influent COD concentrations at the beginning of the cycle resulted in gradients which enabled substrate to diffuse up to the inner core of the granule and therefore larger aggregates could develop. Regarding anaerobic granular sludge at low loading rates hollow cores may develop due to substrate deficiency (Kosaric and Blasczyzk, 1990; Grotenhuis *et al.*, 1991a; Guiot *et al.*, 1992). This phenomenon was normally not observed for aerobic granular sludge, although single granula with a diameter of 5 mm or more showed similar structural deficiencies (Figure 10).

The biomass content varied from 3 to 6 g SS l^{-1}, but increased up to 10-13 g SS l^{-1} under specific conditions (Table 5). A correlation to the process conditions could not be identified. Generally a fast and complete settling was observed for all granular sludges cultivated. The average settling velocity of single granules reached 10 to 40 m h^{-1} at a SVI between 80 and 20 ml g^{-1} (Table 5). In comparison to flocculent activated sludge, granules did not coagulate while settling. Thus, it must be assumed that the surface properties have to be significantly different. An entanglement of filamentous granules or physico-chemical attraction seemed not to influence the sedimentation process.

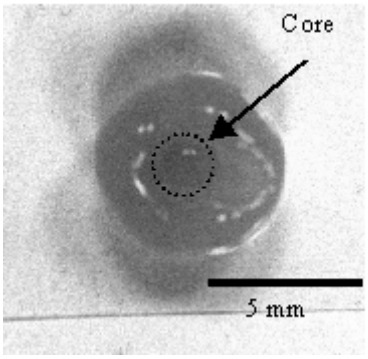


Figure 10. Single Aerobic Granula with a concentric hollow Core

5.1.5 Discussion

5.1.5.1 Granula Formation and Structural Aspects

Generally flocculation and granulation of activated sludge are issues to which no firm methods of characterisation exist. Microorganisms may flocculate due to their own production of extracellular polymers, which tend to encapsulate the cells and cause them to flocculate or to build colonies (Li and Ganczarczyk, 1990; Snidaro *et al*., 1997). Granulation is regarded as a similar process to flocculation.

Although the granulation could be related to an initial selection step to retain mainly biomass with improved settling properties in the reactor, the explicit biological or physico-chemical reasons for the granulation could not be identified. The results indicate that the conditions of the cyclic process were responsible for a granula formation in spherical shape. The substrate loading rate determined the granula growth which apparently was balanced by a detachment process. Fast filling, anaerobic/anoxic phases and periodic feast/famine conditions (kinetic and metabolic selection) limited the growth of filamentous organisms, so that an overgrowth was avoided and the reactors could run in stable conditions for months. Volumetric exchange ratios in the range of 50 to 70 % combined with fast fill and short settling proved to be useful to cultivate aerobic granular sludge in a reproducible way. The contribution of operation conditions, reactor configuration and microbial or genetic factors to the granulation remained unclear. According to the review of Liu and Tay (2002) the granulation seems to be inducible

and hydrodynamic shear forces may play a crucial role in the granulation process. Zhu and Wilderer (2003) used in their experiments very similar process conditions and laboratory SBR compared to this study. The granulation was stable and reproducible, however, the granules were smaller and brown coloured.

If microorganisms observe regularly periods with low or no external substrate available, bacteria that are capable of balancing their growth independent of the external substrate concentration will be enriched (Van Loosdrecht and Heijnen, 1997). Bacteria which are not or only to some extend capable of substrate storage will have to invest extra energy for rapid growth during feast conditions. In famine conditions they will encounter problems in maintaining their cell structure in proper shape. The selected bacteria will have a competitive advantage and keep all cell systems viable even if the external substrate is consumed (Van Loosdrecht and Heijnen, 1997; Van Loosdrecht and Henze, 1999).

Comparing the different process conditions, a significant difference in the granula structure could be observed. Granules cultivated by alternating anaerobic/aerobic conditions were smaller and more compact. According to a general model for activated sludge (Eriksson *et al.*, 1992), there is a tendency that lower growth rates lead to a higher floc strength. Based on this hypothesis the presence of heterotrophic bacteria or PAO might explain the formation of densely structured granules. Generally, autotrophic bacteria and PAO have lower growth rates in comparison to heterotrophic bacteria (Picioreanu *et al.*, 1998a; b).

It is reported that the microbial structure of anaerobic granula was affected by the substrate composition. Dolfing *et al.* (1987) studied the effects of different substrates on anaerobic granules in both upflow reactors and batch reactors. Other substrates than ethanol as carbon source resulted in a higher portion of filamentous bacteria, thus in a higher sludge volume index. Dolfing *et al.* (1987) concluded after parallel experiments that the formation of granules is initially a biological phenomenon, influenced by the choice of substrates. The results of this study partly confirm this hypothesis.

The substrate composition is a determining environmental factor for the developing microbial population, however, it can be a limiting factor for certain species. If granulation is related to certain floc-forming or filamentous organisms, substrate composition could be decisive. However, the microbial composition of the granules resulting from different periods were not compared. The different granules of the periods 2 to 15 might also be explained by changes in the EPS composition of the same microbial community leading to different surface and aggregation properties. One important parameter, for instance, could be varying hydrophobic interactions.

5.1.5.2 Involvement of Filamentous Organisms

Morgenroth *et al.* (1997) suggested a mechanism for the granulation process based on results that emphasise the general role of filamentous organisms. Especially *Geotrichum sp.*, a

filamentous yeast, was isolated in their study. Fungi/yeast were assumed to built a structural backbone of granula. In the present study similar process conditions in the experimental periods 5 to 8 were used, but different carbon sources were chosen. Relatively fast growing heterotrophic bacteria balanced by moderate shear stress, which was only realised by aeration, resulted in granules with a filamentous surface.

Light microscopic observations indicated a dominant structural role of filamentous microorganisms. It is assumed that the filamentous organisms formed the initial aggregates. In accordance with actual models (Sezgin *et al.*, 1978) the entanglement of the filaments inside the granules formed a structural backbone which could resist the shearing forces. Alternating anaerobic/aerobic process conditions repressed fungi, although filamentous bacteria could be observed in the first weeks after granulation. Filamentous organisms may not be a prerequisite for flocculation or granulation, however, in the present study they could be observed for all different process conditions, at least during the first weeks of operation.

5.1.5.3 Effects of the Wastewater Composition

Lettinga and co-workers (1980) recommended the use of wastewater containing carbohydrates for forming anaerobic granules and noted that divalent ions (such as Ca^{2+}) seem to strengthen flocs. Van der Hoek (1987) stated that the stability of aggregates in denitrifying granular sludge in upflow sludge blanket (USB) reactors is largely dependent on the type of substrate used. Changing the carbon source of a reactor that had been fed for 6 months on methanol to glucose resulted in the granular sludge turning filamentous, its settleablilty worsening, and the amount of Ca^{2+}/g TSS decreasing from 216 to 176. He concluded that the granulation was influenced by precipitation of calcium salts. Higgins and Novak (1997b) could show that an increase of the divalent cations (Ca^{2+} and Mg^{2+}) concentration in the feed of the pilot plant was directly followed by an increase of the extracellular protein amount of anaerobic granular sludge.

Ca^{2+}- and Mg^{2+}-ions contribute to self flocculation processes of activated sludge flocs (see section 2.3 and 2.7). It was demonstrated that the concentration of calcium ions in the range of 40-100 mg l^{-1} in the wastewater stream enhanced the rate of sludge aggregation (de Zeeuw, 1981). The aggregates formed in the presence of higher calcium concentrations settled three to four times faster than those at lower concentrations (Mahoney, 1987).

In this study the effect of divalent metal ions was not studied in detail, but parallel experiments with increased Ca^{2+}-ion concentrations did not show an enhanced, faster granulation. The use of tab water with concentrations of 40 to 50 mg Ca^{2+}/l was assumed to affect the aggregation process positively and strengthen the granula structure.

5.1.5.4 Practical Experience

The reasons for the granulation could not be identified. However, volumetric exchange ratios in the range of 50 to 70% combined with fast fill and short settling proved to be useful for a

variety of synthetic wastewater compositions. Although all carbon sources used can be defined as readily biodegradable, wastewater with sucrose prevented granula formation. Granulation was possible for aerobic and for alternating anaerobic/aerobic conditions. Additional to COD removal, enhanced nutrient removal, including nitrification, denitrification, and enhanced biological phosphorus process should be possible to achieve with granular sludge. Batch experiments with aerobic granular sludge indicated the EBPR activity.

The periodic change of feast and famine conditions is believed to be decisive in the granulation process. Several reports indicate that the EPS production or even more important, the EPS surface properties can be modified by cyclic changes in the substrate concentration (Rose, 1984; Chiesa *et al.*, 1985; Rubio and Wilderer, 1987; Flemming and Wingender, 2000). Beun (2001) could show that granulation is possible in continuos airlift reactors, but the granula structure was less compact compared to granules formed in SBR systems.

5.1.6 Summary

- Using SBR systems aerobic granules could be cultivated mainly within 15 and 25 days. Different carbon sources proved to be useful to form granules, however, not all substrates tested allowed this kind of selection and a further granulation process.
- Granula formation can be characterised by two main stages. There was an initial selection step to retain mainly biomass with improved settling properties in the reactor. Determining process parameters for this selection were volumetric exchange ratio and settling time. In the second step small and compact activated sludge aggregates (granules) were formed possibly due to growth and entanglement of filamentous organisms. As a result of the cyclic process, a balance of substrate loading rate and detachment processes granules grew.
- Filamentous organisms, mainly inside the granules, were involved in the initial aggregation step. Fast filling, anaerobic/anoxic selectors and periodic feast/famine conditions (kinetic and metabolic selection) limited the growth of filamentous organisms, so that overgrowth was avoided and the reactors could run for months in stable conditions.

5.2 Molecularbiological and Structural Aspects

5.2.1 Lightmicroscopic Observations

In order to check whether filamentous or higher organisms were present classical lightmicroscopic observations were carried out. Under the studied process conditions (section 4.2, Table 2), typically, in the first weeks after granule formation, filamentous organisms were detected. Up to the sixth week after the first appearance of granules in the reactors granular structure was dominated by morphological different filamentous microorganisms. Figure 11 shows representative filaments at the surface of granular sludge (Table 2, period 10). For a further visualisation of the entangled filamentous matrix, the fluorescence dye SYTO17 was used in combination with confocal laser scanning microscopy (Figure 12).

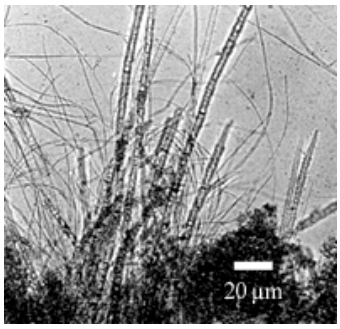


Figure 11. Filamentous Organisms at the Surface of Granular Sludge

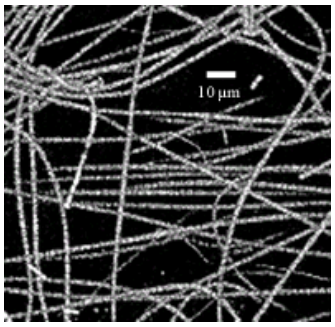


Figure 12. Filamentous Entanglement visualised using SYTO17

An increased sludge age of 15 days or more stimulated the growth of higher organisms. Among others, freely moving and sessile ciliates of the genus *Vorticella* could be detected in high numbers (Figure 13). The significance of protozoa regarding granular formation and structure of granula, and their contribution to the removal capability were not further investigated.

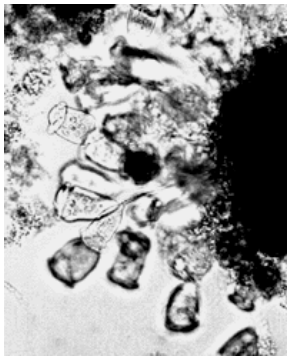


Figure 13. Sessile Ciliates at the Surface of Granular Sludge

5.2.2 Molecular Analysis of the Microbial Community Composition

In close co-operation with the Microbial Ecology Group of Dr. M. Wagner, Institute of Microbiology, Technische Universität München, confocal laser scanning microscopy (CLSM) in combination with fluorescence-*in-situ*-hybridisation (FISH) was used to investigate the microbial population of granular sludge.

The microorganisms present within the granules were characterised using two cultivation-independent approaches. Previously published phylum-, group-, genus-, and species-specific oligo-nucleotide probes were used in the FISH format. This "top-to-bottom"-approach (Wagner *et al.,* 1993; Wagner and Amann, 1997) provides a rapid albeit relatively non-detailed overview of the community composition. Based on these insights, a second approach – the full-cycle rRNA approach (section 2.5.2) – was applied to refine the analysis.

In experimental period 10 (section 4.2, Table 2) sludge samples were taken under steady state conditions. Detailed information on the granula structure could be obtained by embedding and cryo-sectioning of single granules (section 4.6.1). Using an inert aqueous medium cryo-sectioning allows rapid preparation. This procedure allows to retain the original texture and structural heterogeneity of the sample (Flood *et al.*, 2000). Cryo-sectioning has been successfully applied to artificial biofilms (Yu *et al.*, 1994; Zhang and Bishop, 1994). Since the microbial population in sludge aggregates may rapidly respond to environmental changes (Wagner *et al.*, 1993), immediate freezing and/or fixation of the sample was required to prevent any alteration in the population structure.

For FISH investigations granules were analysed using a set of rRNA-targeted oligonucleotide probes, initially (section 4.6, Table 3). Almost all (>99%) of the microorganisms in the granules were detected by FISH. This was demonstrated by simultaneous staining with the EUB338 probe set and the nucleic acid stain SYBR-Green. Ten different granules were studied and at least ten cryo-sections of each granule were used to quantify bacterial groups. The aerobic granular sludge was dominated by *Betaproteobacteria* (88.8 ± 1.6%). In addition, isolated microcolonies of *Gammaproteobacteria* (5.7 ± 3.3%) were observed. Approximately 95% of all microorganisms detected within the granules were *Beta-* and *Gammaproteobacteria*. The quantification of bacterial groups was realised by determining the coverage area in cryo-sections. Using this method small cells might be underestimated (Schmid *et al*., 2000, Daims *et al*., 2001).

Since the reactor showed high phosphate removing activity (chapter 5.5), the aerobic granules were hybridised with probe PAO651 specific for PAO within the *Betaproteobacteria* and the probe ACT1458 specific for the genus *Azoarcus/Thauera* and PAO (section 4.6, Table 3). However, no positive signal could be obtained. In addition, low numbers of filamentous fungi were detected with the *Eucarya* specific probe Euk 516. For further investigation of the diversity of *Betaproteobacteria,* group- and species-specific probes targeting these organisms (Table 3) were applied, but only the *Sphaerotilus natans* specific probe Sna 23a gave a

positive signal with a very small fraction of the microorganisms within the granules. Filamentous microorganisms could be observed by using light microscopy directly after the formation of first granules. In the tenth week after the start of the granule formation, almost no filamentous microorganisms were detected by light microscopy as well as by FISH analysis.

For high resolution analysis of the microbial population structure aerobic granular sludge derived DNA (section 4.2, Table 2, period 10) was used as template for the amplification of almost full length 16S rDNA. 32 clones were sequenced and phylogenetically analysed (Maidak *et al.*, 1996) (Figure 14).

The exclusion of highly variable positions prior to treeing analysis (by the use of a 50% conservation filter for the *Bacteria)* resulted in identical assignment of the clone sequences (data not shown). Bootstrap support (data not shown) for the clustering of the granular sludge retrieved sequences with the respective reference organisms is highly significant (Figure 14). Bootstrap values were determined by the maximum parsimony method. The 32 analysed clones provide a good estimate of the diversity covered by the established library. This was demonstrated by estimating the diversity coverage and by rare faction plotting. For both types of analysis the 32 clones were classified into operational taxonomic units (OTUs) which encompass obtained sequences with a similarity of 97% or more. Applying this criterion, the 32 molecular isolates from the granules could be assigned to 10 OTUs. Six out of ten OTUs consisted of only one clone. Consequently, the 32 analysed clones represent 81% of the OTUs within the library according to the following formula:

$$C_{coverage} = [1-(n1/N)] \times 100\% = [1-(6/32] \times 100\% = 81\%$$

n1 = number of OTUs represented by only a single clone
N = total number of clones analysed

This coverage is much higher than those usually obtained in 16S rDNA cloning analysis of complex microbial communities which indicate a relatively limited species diversity in the aerobic granules. A significant increase of the coverage would require the analysis of a high number of additional clones as visualised by rare faction plotting (Figure 15).

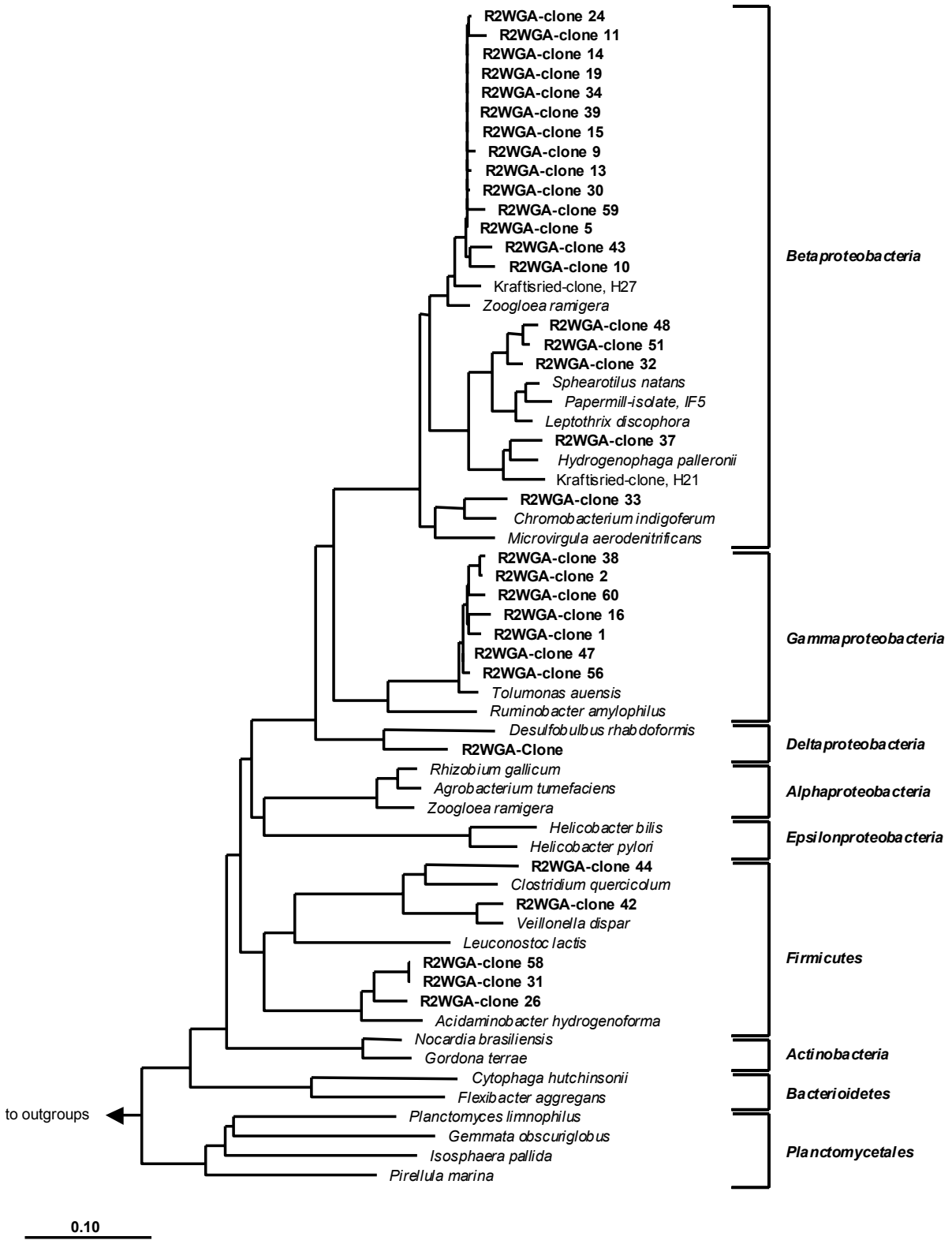


Figure 14. Phylogenetic 16S rDNA Tree Reflecting the Relationship of 32 Aerobic Granule Clones (labeled R2WGA) and Reference Organisms Belonging to Different Phylogenetic Groups (Indicated by Brackets) within the *Bacteria*. The Bar Represents 10 % Estimated Sequence Divergence.

The phylogenetic 16S rDNA tree (Figure 14) was constructed by using a maximum-likelihood tree which was calculated exclusively with full-length 16S rRNA sequences as backbone. Partial 16S rRNA sequences were subsequently added using the ARB parsimony tree without changing the overall tree topology.

Most of the clones were affiliated to the type strain of *Zoogloea ramigera*^{T}. This was surprising, since after the application of the oligonucleotide probe Zra 23a (Table 3) described to be specific for *Zoogloea ramigera*^{T} no positive hybridisation signal within the granules was observed. Analysis of the *Zoogloea ramigera*^{T} affiliated clone sequences revealed, that none of them possessed a fully complementary target site of probe Zra 23a (Rosselló-Mora *et al*., 1995). In order to be able to detect the entire *Zoogloea ramigera*^{T}-cluster, including the newly determined granule clones via FISH, the recently designed oligonucleotide probe S-*-Zora-1414-a-A-20 specific for *Zoogloea ramigera*, and affiliated clone-sequences were applied (Table 3, Juretschko *et al.*, 2002). *In situ* hybridisation with probe S-*-Zora-1414-a-A-20 revealed that *Zoogloea ramigera*^{T}-like organisms represent the majority of the bacterial population of the aerobic granules (88.4 +/- 4.1%, Figures 16 and 17).

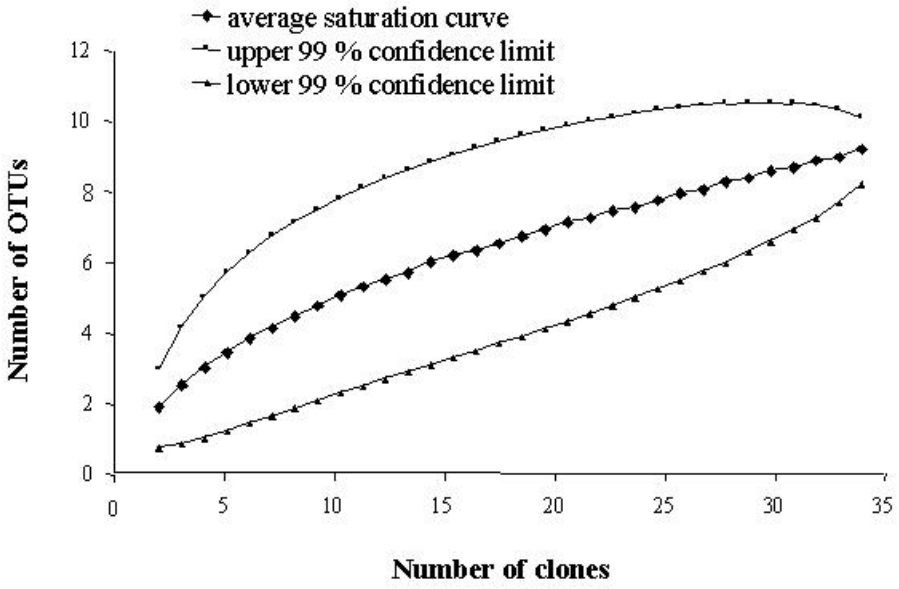


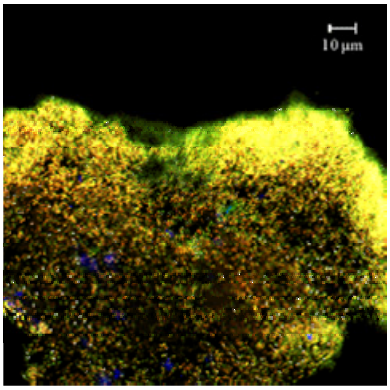
Figure 15. Saturation Curve of a Rarefaction Analysis of 34 Clones from Aerobic Granules.

The upper and lower 99% confidence limits in Figure 15 are indicated by squares and triangles, respectively. The calculation was done by the shareware program *aRarefactWin* (Analytical Rarefaction 1.2; Holland, 1998).

As visualised by *in situ* hybridisation of granules (Figure 16, 17) microcolonies of *Gammaproteobacteria* appear within the zoogloeal cell matrix. Since the 16S rDNA clone library of the aerobic granules also contained several gamma-subclass 16S rDNA sequences related to *Tolumonas auensis* (see Figure 14), probe S-*-Clta-0467-a-A-18 specific for the *Tolumonas auensis* affiliated clones was designed. Probe S-*-Clta-0467-a-A-18 had one

mismatch to *Tolumonas auensis* and at least three mismatches to all other 16S rDNA sequences in the database (Figure 18).

In Figure 16 and 17, *Betaproteobacteria* appear green (Bet 42a labelled with Fluos, Table 3). *Zoogloea ramigera*-like organisms (probe S-*-Zora-1414-a-A-20 labelled with Cy5, red colour) appear yellow due to the overlay of green and red. *Gammaproteobacteria* (Gam 42a labelled with Cy3), in a cryosection of an aerobic granule, are visible in blue.



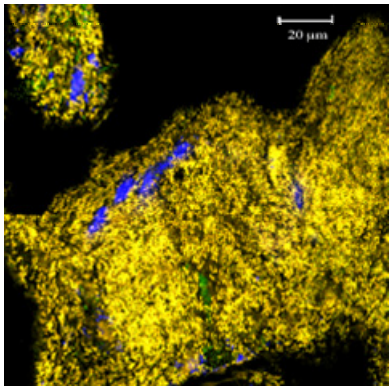


Figure 16 and 17. *In situ* Identification of *Zoogloea ramigera*-like Organisms, *Betaproteobacteria* and *Gammaproteobacteria* in Cryosections of Aerobic Granules.

Since no pure culture was available to determine the optimal hybridisation stringency for probe S-*-Clta-0467-a-A-18, the *in situ* probe dissociation curve was recorded with fixed cryo-sections of the aerobic granules (Figure 19). The probe yielded strong signals up to 15% (v/v) formamide in the hybridisation buffer followed by a decline at 20% (v/v) formamide (Figure 19). Based on the obtained results, a formamide concentration of 10% (v/v) was chosen as optimal for probe S-*-Clta-0467-a-A-18.

```
Probe sequence                 3'-TGCGTAGTTGTAACTGCA-5'
Target sequence                5'-ACGCAUCAACAUUGACGU-3'
  R2WGA-clone 16                 -..................-
  Tolumonas auensis              -........U.........-
  Aeromonas media                -........G.UG......-
  Aeromonas eucrenophila         -........G.UG......-
  Aeromonas sp. 'CDC 859-83'     -........G.UG......-
  Microscilla marina             -G.......G..A..G..C-
  Flexibacter flexilis           -G.......G..A..G..C-
  Persicobacter diffluens        -G.......G..A..G..C-
```

Figure 18. Difference Alignment for Probe S-*-Clta-0467-a-A-18. 16S rRNA Sequences are Displayed for Representative Reference Organisms. The R2WGA-clone 16 was Selected as a Representative for all 7 Clones Derived from the Aerobic Granules and Affiliated to *Tolumonas auensis*.

The relative strength of the hybridisation was determined at increasing concentrations of formamide in the hybridisation buffer and decreasing concentrations of NaCl in the washing buffer by a quantification of intensities of the fluorescent signal (Figure 19). *Aeromonas media*, used as negative control, showed signal intensities similar to the autofluorescence under all formamide conditions tested (data not shown).

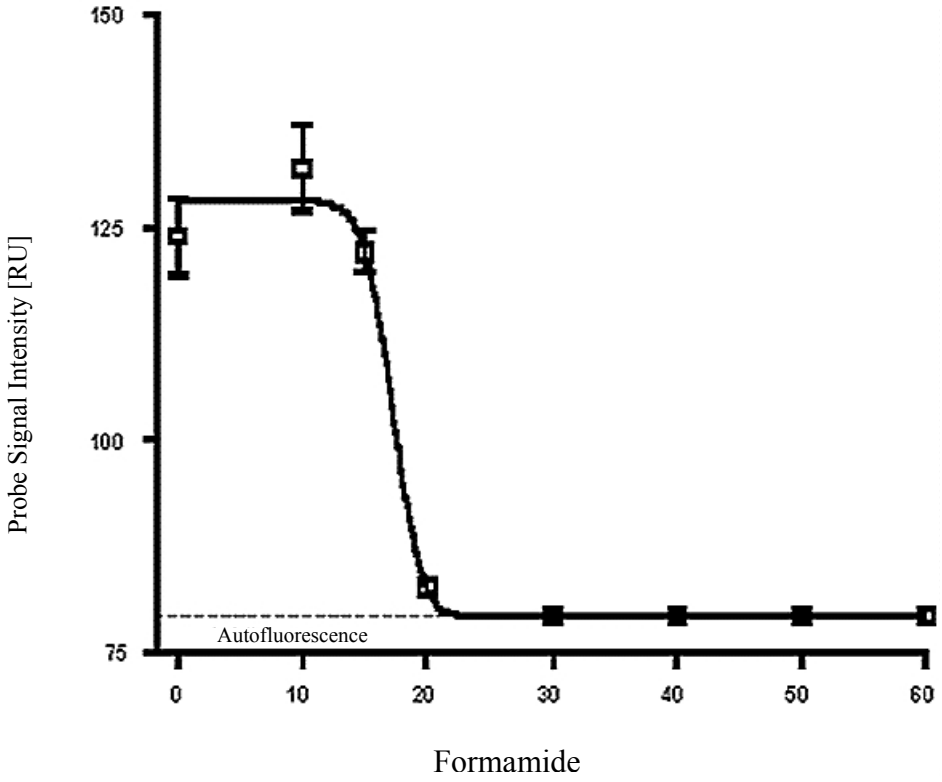
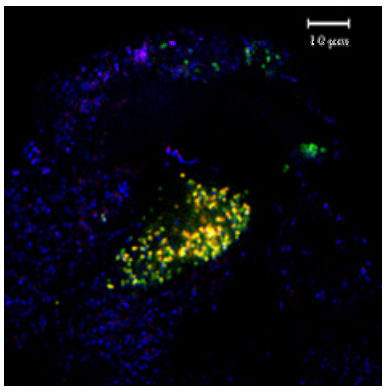


Figure 19. Probe Binding Profile of Probe S-*-Clta-0467-a-A-18

Hybridisation with probe S-*-Clta-0467-a-A-18 confirmed that the *Tolumonas auensis*-like organisms are forming cell clusters of *Gammaproteobacteria* within the aerobic granules. In Figure 20 and 21 *Tolumonas auensis*-like organisms appear in purple due to simultaneous hybridisation with probe S-*-Clta-0467-a-A-18 (labelled with Cy3), Bet 42a (Table 3) specific for *Betaproteobacteria* (labelled with Fluos, here green) and Gam 42a (Table 3) specific for *Gammaproteobacteria* (labelled with Cy5, here blue).



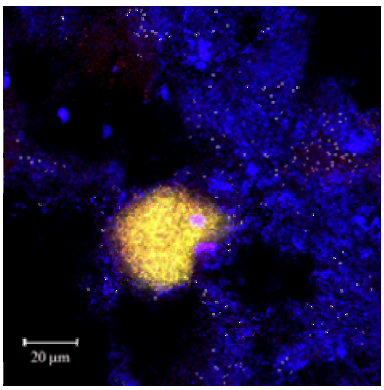


Figure 20 and 21. *In situ* Identification of *Tolumonas auensis*-like Organisms, *Betaproteobacteria* and *Gammaproteobacteria* in a Cryosection of an Aerobic Granule

5.2.3 Ribosome Content

In order to link the distribution of active microorganisms with the architecture of the granules, the ribosome content of the microorganisms, in relation to their spatial occurrence within the granule, were determined. Therefore, granular sludge was cryo-sectioned.

From the cryo-sections of granula high resolution CLSM images (2048x2048 pixel) were acquired after FISH and SYBR-Green DNA staining. The images were analysed by two newly macros (developed by the Microbial Ecology Group of Dr. M. Wagner) for the Kontron KS400 digital image analysis software package from Carl Zeiss Vision. While both macros split the high resolution images into squares of 256x256 pixels, the macro "Split Image Intensity Measurement" (SIIM) measures the mean intensity of these squares (reflecting the number of ribosomes) and the macro "Split Image Area Measurement" (SIAM) calculates the ratio of stained pixels to the total amount of pixels (reflecting the packaging density of the cells).

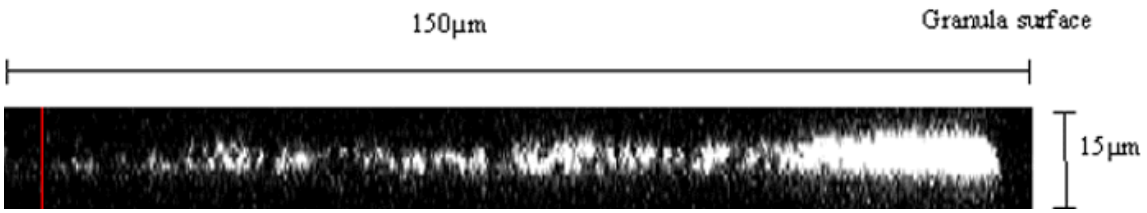


Figure 22. CLSM Z-Section of a Granule Cryo-Section (15 µm thick) hybridised with EUB Probe Mixture. Compression of Cryo-Section in Z-Direction in the inner Parts of the Granule is due to collapsing hollow Cavities during the Dehydration Step

By using the SIIM macro it could be demonstrated that the cells in a layer at the surface of the granules show the highest ribosome content. This indicates maximum activity at the surface of the granules (Figure 23). In Figure 23, granule cryo-section was hybridised with the bacterial probe set and subsequently recorded using CLSM. The square with the highest intensity was set to 100%. Square intensities are indicated by gray values, (bottom left) leading to a display of intensity (ribosome content) distribution (bottom right).

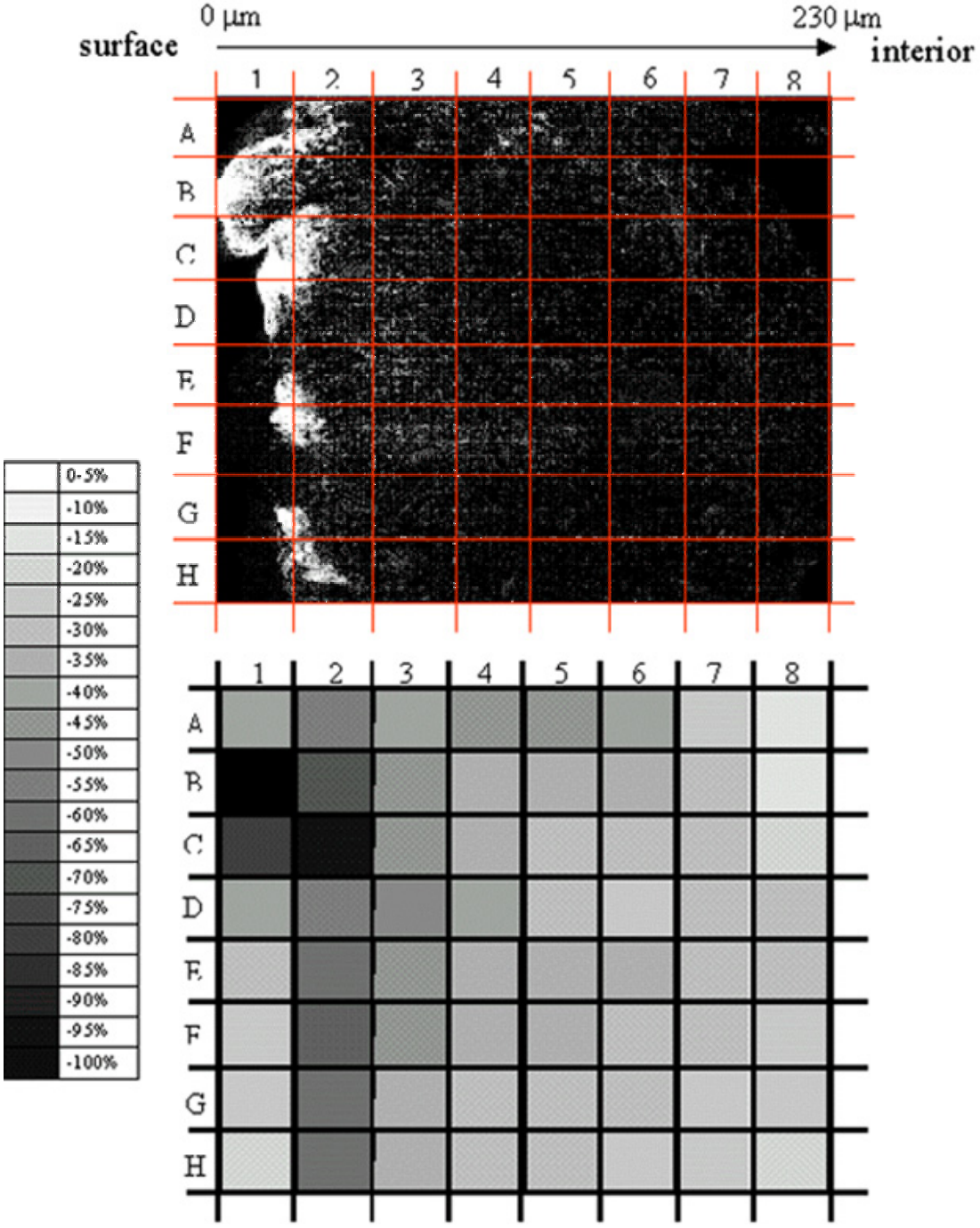


Figure 23. Ribosome Content of microbial Cells in relation to their Spatial Distribution within the Granule. The Image is Split in Squares of 256x256 Pixels (top) and the average Intensity of these Squares is Measured using the SIIM Macro.

Deeper inside the granules the bacteria possess a drastically reduced ribosome content, possibly caused by limited substrate availability. Maximum cell densities were recorded at the surface region of granules (Figure 24). This is in accordance with the results regarding high surface activity. In Figure 24, a granule cryo-section was stained with DAPI and subsequently recorded using CLSM. The image is split in squares of 256*256 pixels (top). The square with the highest density was set to 100 %. Square densities are indicated by gray values, (bottom left) leading to a display of density distribution (bottom right).

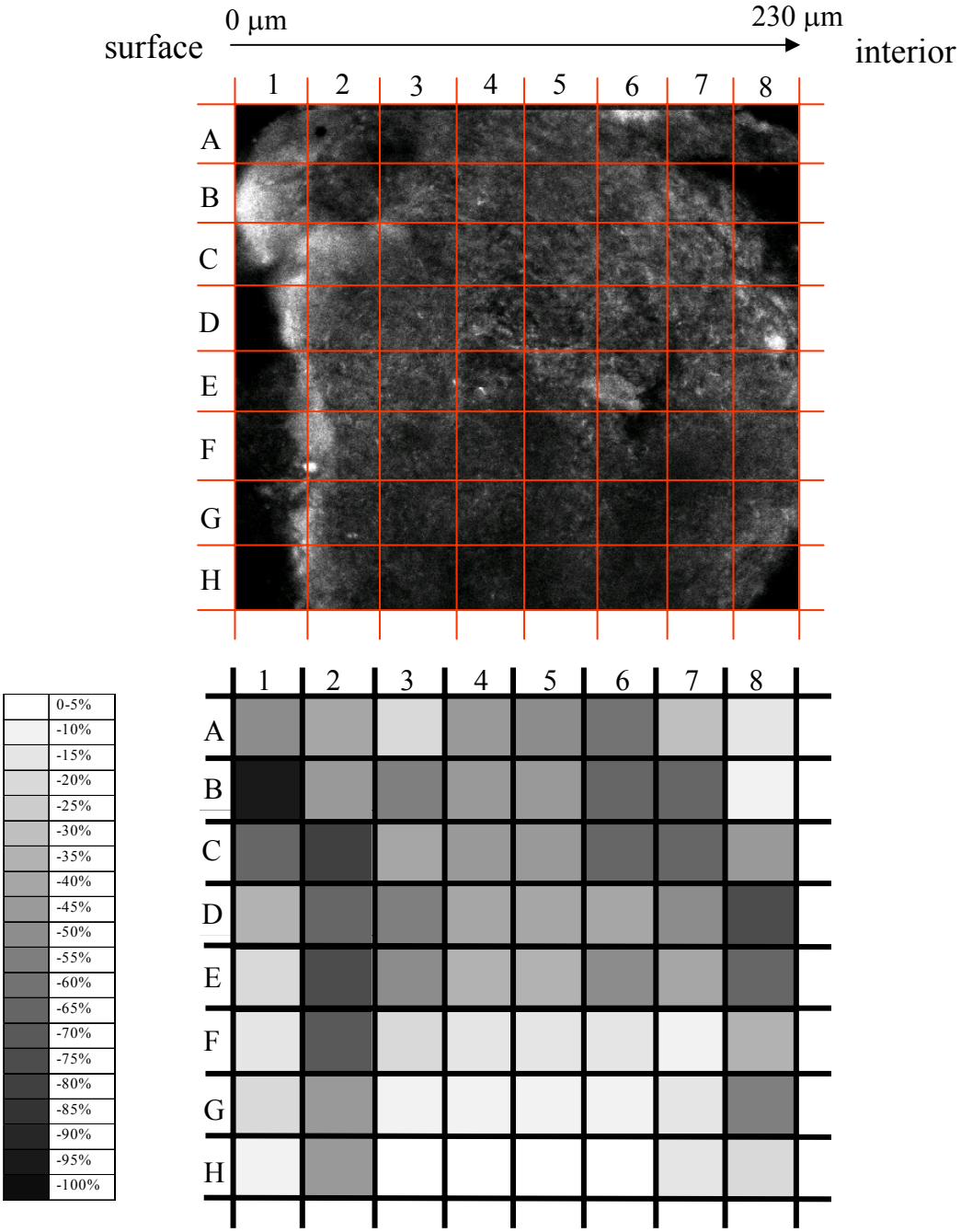


Figure 24. Density of microbial Cells in relation to their Spatial Distribution within the Granule. The Image is split in Squares of 256x256 Pixels.

5.2.4 Discussion

5.2.4.1 Molecular Microbial Population Analysis of Aerobic Granules

Aerobic granular sludge showed efficient and stable COD removal combined with the capability for enhanced nutrient removal. Nitrification and simultaneous denitrification as well as EBPR were observed (see chapter 5.5). In the present study *in situ* hybridisation techniques in combination with AOB-specific oligonucleotide probes were used to detect AOB (Ammonia Oxidizing Bacteria) in the granules.

Purkhold *et al.* (2000) presented a summary of hybridisation probes for beta-subclass AOB. Their work indicated that the probe Nso 1225 targets allmost all ß-AOB. However, no positive signals for ß-AOB could be identified using oliginucleotide probe Nso1225. Further investigations are necessary to answer the question if heterotrophic nitrification might be responsible for the measured nitrification.

EBPR is achieved by selecting for PAO and can be advantageous over chemical phosphorus removal, since e.g. no chemicals are required. No signal could be obtained by using FISH with oligonucleotide probe PAO651 specific for known typical PAO (Crocetti *et al.,* 2000). This leads to the conclusion, that other bacteria than the previously proposed poly-phosphate accumulating bacteria (Hesselmann *et al.*, 1999; Crocetti *et al.,* 2000) were involved in this process.

In accordance with previous investigations of municipal activated sludge systems (Wagner *et al.,* 1993, Wagner *et al.,* 1994; Kämpfer *et al.,* 1996; Wagner *et al.,* 1997, Juretschko *et al.,* 1998), the aerobic granules were dominated of *betaproteobacteria*. As reflected in more detail by the 16S rDNA clone library with a coverage of 81% (Figure 14) and fluorescence *in situ* hybridisation (Figure 16,17), respectively, this very large fraction of *betaproteobacteria* consisted almost exclusively of *Zoogloea ramigera*^{T} related organism. Embedded within this zoogloeal cell matrix were *Tolumonas auensis* (Fischer-Romero *et al.*, 1996) related cell clusters (Figure 20, 21). While *Zoogloea ramigera*^{T} is known as EPS producer (Farah and Unz, 1976; Unz 1989; Dugan, 1992), the role of *Tolumonas auensis* related organisms within the aerobic granules needs to be further investigated.

5.2.4.2 The Role of *Zoogloea ramigera*^{T} related Cells in Granule Formation

Notably, almost no filamentous bacteria, which were present in the first weeks after formation, were detected in the tenth week after granule formation. According to conceptual floc models, filamentous bacteria can build the backbone and first possible attachment sites for EPS producing bacteria like *Zoogloea ramigera*^{T} related cells (Jenkins, 1986) (see also chapter 2.3). However, within the aerobic granules filaments seemed to be out-competed after a few weeks and the overall granule structure consisted mainly of zoogloeal cells.

As shown in Figure 23, the bacterial community mainly consisted of *Zoogloea ramigera*^{T} affiliated organism, which were forming two layers within the aerobic granule. The surface consists of highly active and densely packed cells while the core region of the granules is a loose structure with holes and channels, which are probably a result of cell decay (Figure 22). In comparison to anaerobic granules, which possess a clearly defined structure of cortex and medulla (Macario *et al.*, 1991), the not so well structured aerobic granule posses various ecological niches. Such niches might be the presupposition to use granular sludge for improved bioaugmentation purposes as shown for anaerobic granules (Ahring *et al.*, 1992; see chapter 6).

5.2.4.3 Fluorescence-*In*-*Situ*-Hybridisation

FISH, as a modern scientific method in wastewater science and technology (Wilderer *et al.*, 2002), was used since a direct assessment of species by FISH avoids averaging of the sample. This provides very precise information of the nature of the individual populations at high resolution (Wagner *et al.*, 1997). FISH probing of rRNA can be used to identify individual cells at any phylogenetic scale, from the broad domain level down to the subspecies strain. In general, hybridisation-conferred fluorescence is scored on a positive or negative basis for each cell. However, some investigators have used fluorescence intensity measurements to estimate ribosome concentrations within a cell and extrapolate this as an indicator of cellular activity (Poulsen *et al.*, 1993). The correlation between activity and rRNA content, however, may be species-specific (Hsu *et al.*, 1994; Fukui *et al.*, 1996).

Of the environmental studies that have addressed sludge aggregates or biofilms, most have relied on traditional microbiological techniques, such as heterotrophic plate counting after homogenisation (Flood *et al.*, 2000). Dispersion and culturing techniques cannot record aggregate morphology and introduce a culture-dependent bias into population analyses, thus rendering them largely unsuitable for environmental applications (Wagner *et al.*, 1993; 1997). In contrast CLSM in combination with FISH of sludge aggregates showed heterogeneous structures of cell clusters interspersed with voids and channels (Lawrence *et al.*, 1991; de Berr *et al.*, 1993b; Stoodley *et al.*, 1994). These structures have been shown to alter with species composition and environmental conditions (Lawrence *et al.*, 1991; Huang *et al.*, 1995; Møller *et al.*, 1997; Stoodley *et al.*, 1997).

5.2.5 Summary

With the initial use of FISH, using phylum-, group-, genus-, and species-specific oligonucleotide probes to determine the microbial community structure, it became evident, that the population consisted mainly of *betaproteobacteria* (approx. 89%) and *gammaproteobacteria* (approx. 5%).

For a more detailed view on the population structure, the full-cycle rRNA approach was applied. 21 of 32 16S rDNA clones of the aerobic granules were affiliated to *Zoogloea*

ramigera^T within the *Betaproteobacteria* (14 clones) and to the *Gammaproteobacterium Tolumonas auensis* (7 clones). After construction and application of species and clone specific oligonucleotide probes, it turned out, that *Zoogloea ramigera* (approx. 88%) and *Tolumonas auensis* (approx. 5%) are the most abundant species in the aerobic granular sludge. Although no ammonia- and nitrite-oxidising bacteria were detected, the granules showed efficient ammonium removal activity (see chapter 5.5).

In conclusion, the analysis revealed novel insights into the microbiology of well-settling aerobic granules capable of nitrogen and phosphorous removal. In brief, the results:

- The major component of these granules was a typical floc-forming bacteria, *Zoogloea ramigera*^{T}. In addition, *Tolumonas auensis*-like bacteria were present in significant amounts. None of these species has been reported to be involved either in nitrification or biological phosphorous removal.
- The full cycle 16S rDNA approach indicated a relatively limited species diversity which might be related to the use of readily biodegradable substrates as C-sources.
- Light-microscopic observations and the molecular-biological results showed that filamentous organisms were involved in the initial aggregation step. The filamentous backbone of the initial granules was replaced by an extracellular polymeric structure, possibly of floc-forming bacteria.
- A shift of the microbial population of the granules is possible. Otherwise the morphology of the microorganisms in the initial granules changed.
- Higher organisms, mainly freely moving and sessile ciliates, could be observed, however, their significance regarding the formation and structure of aerobic granules remained unclear.
- Cavities in the inner region of the granules, which are probably formed by cell decay, could be identified by *in situ* hybridisation (Figure 22). The channels and holes caused shrinkage of the inner granule region during the dehydration step of the hybridisation procedure.

5.3 Phyisco-chemical Properties

Activated sludge is well investigated and data about particle size, wet density and settling properties of activated sludge flocs are easily available. This information is important in general for the study of mixed liquor suspended solids, especially for design procedures of sedimentation basins. Since aerobic granular sludge is yet only rarely documented in the literature (section 2.7.3) important data is missing. One determining parameter for the substrate diffusion is the aggregate porosity (Li, 1987). Moreover the porosity influences the settling characteristics. Diameter, density and final settling velocity were examined to estimate the granular sludge porosity by modified Stokes` equations.

Adsorption is an important regarding mass transfer, because particles and molecules are fixed by this physico-chemical process at the sludge surface at which reactions take place. The adsorption capacity of activated and granular sludge was compared by determining the specific surface area of the sludge. Finally the shear sensitivity of granular sludge was characterised in batch tests.

5.3.1 Porosity Estimation

5.3.1.1 Determination of Settling Velocity and Size

In free settling tests using a plastic cylinder the settling time of single granules was taken. All tests were performed twice and the average was determined. Aerobic granula were directly after each settling test photographed and the average granula diameter was determined. A total of 78 granules of each experimental period were examined. By means of digital image analysis aggregate diameters were determined granules were assumed to be spherical. For granules of the period 2 (section 4.2, Table 2) a diameter range from 1.5 to 4.9 mm was observed. Final settling velocities from 0.35 cm/s to 1.80 cm/s were reached, corresponding to Reynolds number ranging from 0.006 to 0.75 ($Re = d_g \times \rho_w \times \nu / \eta$). The average diameter was 3.2 mm. The average settling velocity was 0.97 cm/s. In Figure 25 granule diameter is plotted versus terminal settling velocity. A general trend could be observed that, increasing aggregate diameter results in a higher terminal settling velocity in agreement with Stokes` law.

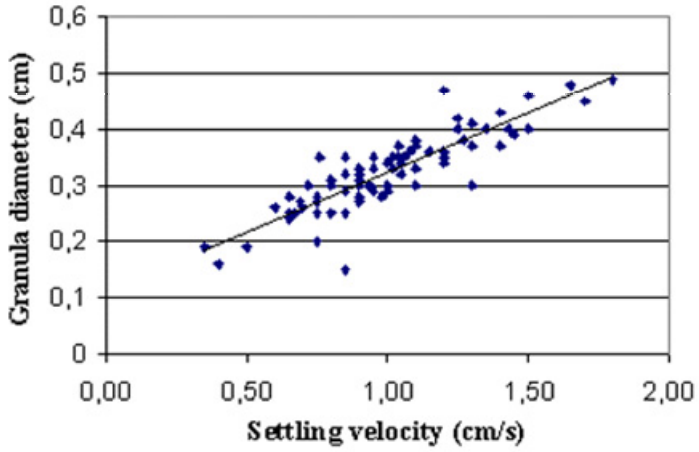


Figure 25. Granula Diameter as a Function of Terminal Settling Velocity

For granules of period 15, diameter ranged from 1.1 to 6.5 mm and the average was 3.0 mm. Final settling velocities up to 2.00 cm/s could be observed and the average was 1.05 cm/s. Equal to the trend in period 2 (Table 2) increasing aggregate diameter resulted in a higher terminal settling velocity (Etterer and Wilderer, 2001).

5.3.1.2 Isopycnic Density Measurement and Porosity Calculations

In four following weeks of period 2 (Table 2), 80 aerobic granules of the same reactor were analysed. For each measuring day the density of 20 granules was determined and the average was calculated. The average density of all examined granules was 1.044 g/ml. Results are presented in Table 6, which lists the density variations over time and the density range of each measuring day. Density at a given time was within a small range regardless a general trend can be identified. This trend indicates, that the average density is slightly decreasing over time.

Table 6. Density of Aerobic Granular Sludge over Time (Period 2, Table 2)

Day of Measurement	Average Density (g/ml)	Density Range
1.	1.047	1.046-1.049
8.	1.050	1.049-1.050
17.	1.042	1.041-1.044
26.	1.038	1.037-1.039
Total average	1.044	

According to Gregory (1997) aggregation of particles usually gives self-similar, fractal structures with a decreasing density with size. Therefore the measured densities of granules as a function of their diameter is given in Figure 26. There was no significant trend recognizable.

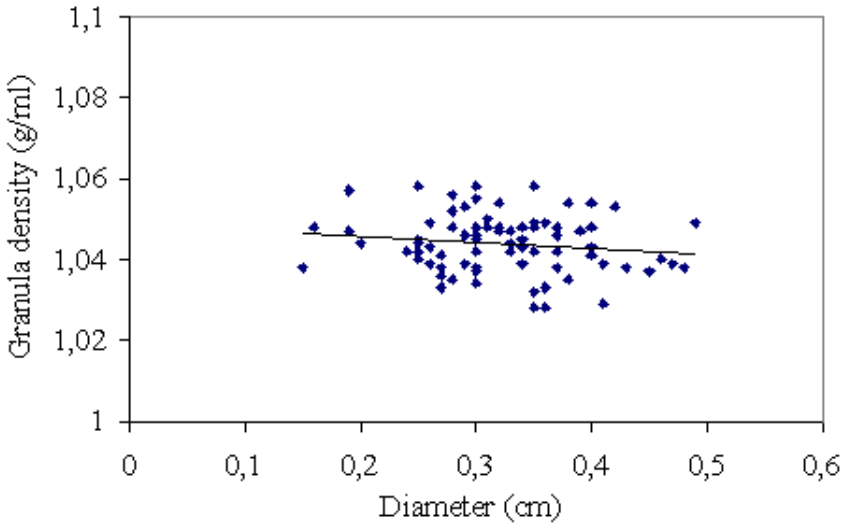


Figure 26. Granula density as a function of granula diameter

Also during the second run the density of the granular sludge was analysed. For each measuring day the density of 20 granules was determined and the average was calculated. The total average density was 1.048 g/ml. Results are presented in Table 7, which lists the density variations over the time and the density range of each measuring day. The average density decreased over time.

Table 7. Density of Aerobic Granular Sludge over Time (Period 15, Table 2)

Day of measurement	Average density (g/ml)	Density range
1.	1.053	1.050-1.054
7.	1.050	1.048-1.052
16.	1.045	1.043-1.047
27.	1.043	1.040-1.044
Total average	1.048	

In the calculation of granula porosity it is assumed that primary particle diameter is 10 µm. Primary particle are the smallest units of a floc and their diameters range from 1 to 20 µm (Lee *et al*., 1996). Solid volume fraction, necessary for permeability models, is constant at a level of 0.2. Bound water content of activated sludge measured by Lee and Hsu (1995) was taken as an approximation for primary particle density. With these parameters and the determined average settling velocity, a typical granula porosity was calculated from Equation (1) (section 4.6.1). The estimation gives a porosity for aerobic granula of 72 % for granules of period 2 (Table 2) and a averaged porosity for the granular sludge of period 15 of 65 % (Etterer and Wilderer, 2001).

5.3.2 Discussion and Summary

To investigate wet density of aerobic granular sludge, isopycnic centrifugation was chosen as a method to determine aggregate density. The densities of aerobic granules were not stable with time. The listed results in Table 6 and 7 show a slight general trend of decreasing densities. Since the accuracy for isopycnic density measurement is ± 0.001 g/ml under favourable conditions (Dammel, 1991), the observed variations can not be explained by measuring inaccuracy. Hence reasons for the density decrease have to be identified. One could be the fact, that fractal aggregates show a decrease in density with an increase of the diameter. Activated sludge flocs and also aerobic granules are fractal aggregates and during the period of nearly four weeks average diameters of all granules in the reactor were growing. Based on this fact decreasing densities can be explained by the duration of the measuring period, but from a statistical point of view more measuring series are necessary to prove this hypothesis. The total average density of granula is within the range of the densities of activated sludge flocs from different waste water treatment plants. Dammel measured a density range of 1.036-1.062 g/ml for activated sludge flocs. So the wet density of aerobic granules is not deviating significantly from activated sludge flocs.

Additionally the porosity of aerobic granules was estimated based on free-settling tests. Some assumptions had to be done. This means that, the density of primary particles can vary in the range from 1.08 -1.40 g/ml. The density of dried solids could be taken, but this parameter can only function as an upper limit for the primary particle density. For this reason estimations based on Equation (2) were used. Factor Ω is determined by a permeability model and different models result in a varying aggregate permeability (Lee, 1996). For this study only Brinkman model was used. Another uncertainty arises from the possibility of flow through porous aggregates. Some authors (Li, 1988; Gregory 1997) discuss flow through flocs and other permeable aggregates and this seriously influences the drag coefficient. If flow through porous media takes place in general the drag coefficient significantly increases. Despite all assumptions reasonable average porosity values of 65 and 72% could be calculated.

Principal objectives of this study were the investigation of parameters that are characteristic for granular sludge settling, and the following conclusions were reached:

1. Aerobic granules were not significantly more dense than activated sludge flocs.
2. Settling velocity of single granules could be described by modified Stokes` law.
3. Due to an increased granula diameter and possibly a lower drag coefficient compared to activated sludge flocs, granules showed significant higher settling velocities up to 2.00 cm/s.
4. Calculations based on free-settling tests resulted in an approximation for the aggregate porosity. Values of 65 and 72% were determined.

5.3.3 Adsorption Capacity

5.3.3.1 Adsorption and Porosity

Activated sludge aggregates, are multiphase systems that consists of solids and of a liquid phase. Biofilms are spatially heterogeneous, characterised by complex assemblages of organisms and spatial gradients of physical/chemical parameters (Hamilton *et al*., 1989), which can surely be stated for suspended sludge, too. As a result of these spatial gradients, the microbial species, the porosity and the density will vary in different depth of the floc or granula. These spatial distributions of biotic and abiotic components in turn affect the mass transfer (Sun et al., 1989; Fan et al., 1990; Zhang and Bishop, 1994a, b).

Smith and Coackley (1984) showed that dye adsorption tests can be useful to study the adsorption capacity and the pore structure of activated sludge. They reported that the surface area of porous solids can be determined by dye adsorption techniques. The used dye Lissamine Scarlet 4R, whose colour index generic name is Acid Red 18 (AR18), has been reported to be suitable, since a monolayer adsorption can be assumed. Therefore to further characterise the structure and adsorption capacity of granular sludge in comparison to flocculent activate sludge dye adsorption tests using AR 18 were carried out.

Empirical factors are often used to quantify the nature of porous solid. The mean pore radius r is one such factor and its definition by Satterfield (1970) can be adapted for activated sludge (Smith, 1981), thus:

$$r = \frac{2.5 \cdot \varepsilon}{S \cdot D_W} \tag{4}$$

where S = surface area per unit mass of porous media ($m^2\ g^{-1}$)

D_W = wet density of the sludge ($g\ cm^{-3}$)

ε = porosity of sludge aggregate (%)

r = mean pore radius (μm)

The factor of 2.5 in equation 4 is derived from assuming pores are cylindrical and spherical (Broeckhoff and Linsen, 1970). A factor of 2 represents cylindrical pores and purely spherical pores would give a factor of 3. For a given density and porosity of sludge the mean pore radius will be inversely proportional to the specific surface area.

The surface area of porous solids can be measured by dye adsorption techniques. A method for applying these techniques to activated sludge has been discussed by Smith and Coackley (1983). According to their study surface area of sludge was calculated as follows:

$$S = Y \times N \times A \tag{5}$$

where S = specific surface area ($m^2 g^{-1}$ TS)

Y = adsorbed dye (mol g^{-1})

N = Avogadro's number (6.023×10^{23})

A = area covered by each molecule (m^2 mol^{-1})

The maximum area covered by each molecule of Acid Red 18 is 196 $Å^2$ (Smith and Coackley, 1983).

5.3.3.2 Determining the Specific Surface Area

To determine the dye adsorption equilibrium granular sludge suspension was filled in six different flasks and the adsorption of the remaining dye in solution was determined after 30, 60, 120, 180, 480 min and 24 h. According to the described test procedure (section 4.8.3) samples were centrifuged and analysed. Results indicate that the dye adsorption equilibrium was achieved within 30 min (Table 8), although there were slight adsorption increases after 2 and 3 hours of incubation. Smith and Coackley (1983) noted an adsorption equilibrium for activated sludge flocs of 30 min.

Table 8. Dye Adsorption Equilibrium Test with Aerobic Granular Sludge

Sample	1	2	3	4	5	6
Time of contact (h)	0.5	1	2	3	8	24
Absorption	0.12	0.12	0.13	0.12	0.12	0.12
Dye in solution (mg l^{-1})	4.7	4.7	5	4.9	4.7	4.7

Dye adsorption is often pH dependent. Longmuir (1975) found that adsorption of Acid Red 18 on activated sludge was taking place only under acidic conditions. Under neutral pH conditions the electrostatic repulsion between the anionic dye molecule and the negatively charged sludge surface is responsible for the lack of adsorption. As the pH is lowered, however, the charge of the sludge surface drops to zero or possibly becomes positive. A pH of 2.5 was found to be suitable for the used dye (Smith and Coackley, 1983). Therefore the pH was adjusted to 2.5 and a calibration curve in the range between 0 and 120 mg Acid Red 18 l^{-1} was measured. Adsorption at 505 nm showed to be linear related to the concentration of Acid Red 18 (Figure 27).

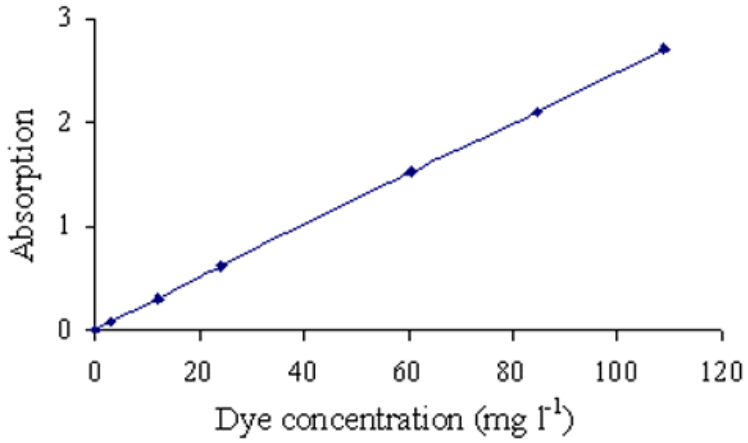


Figure 27. Calibration curve of Acid Red 18 at pH 2.5 (505 nm).

Two isotherms are shown in Figure 28 which were assumed to be representative for the adsorption properties of flocculent and granular sludge (period 10, Table 2). The ordinate was plotted as mmol dye adsorbed per kg suspended solids. The abscissa represents the concentration of remaining adsorbate in solution at equilibrium. Granular sludge adsorbed relatively quickly a maximum of 133 mmol dye kg^{-1} solids. In comparison activated sludge flocs could adsorb 141 mmol dye kg^{-1} solids. The maximum dye adsorption of both sludge samples was very similar.

The curves (Figure 28) imply that there was high affinity of the dye towards the sludge. Up to a certain limit, all the dye was adsorbed by the sludge leaving none in solution. Ion-exchange or chemical bonding could be responsible for this behaviour. This chemisorption is usually about 40-60 mmol dye kg^{-1} solids (Smith and Coackley, 1983). The shape of the isotherm indicates that a second layer on top of the chemisorbed material is being formed due to physical adsorption of dye molecules. According to earlier studies the observed isotherm is the sum of two isotherms, one dependent and one independent of the concentration of solution (Smith and Coackley, 1983; Zhang and Bishop, 1994b) between the dye molecules and the sludge surface.

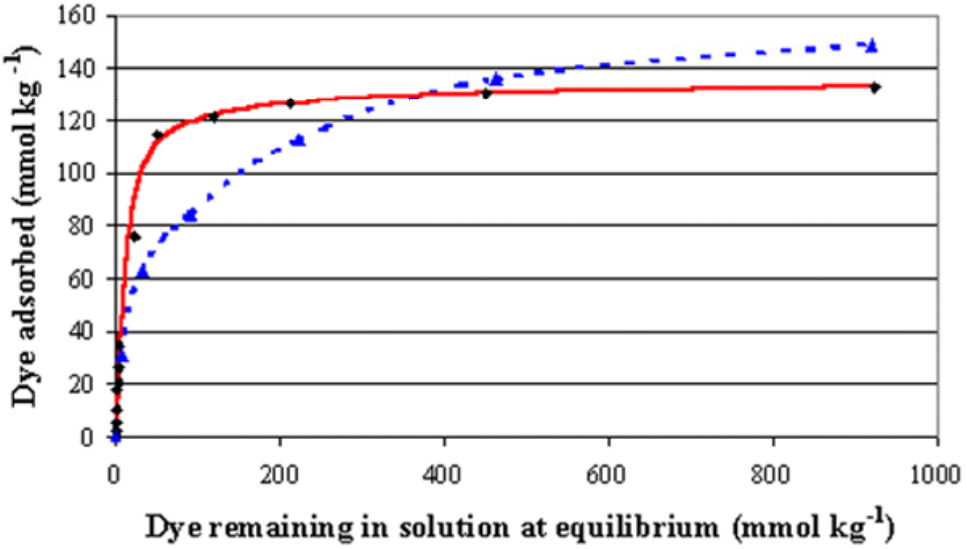


Figure 28. Dye adsorption curves for activated (dotted line) and granular (bold line) sludge using Acid Red 4R dye

Although the determination of specific surface area was experimentally convenient it cannot be considered free of systematic errors. It has to be assumed that a monolayer of adsorbed dye was existing. Based on a linear relationship between sludge density and the maximum dye adsorption quantity (Smith and Coackley, 1984; Zhang and Bishop, 1994b) the specific surface area of flocculent and granular activated sludge were calculated using Equation (5).

Table 9. Specific Surface Area of Flocculent and Granular Sludge

	Flocculent Sludge	**Granular Sludge**
Quantity of adsorbed dye (Acid Red 4R) Y (mol g^{-1})	141	133
Area covered by each molecule A (m^2 mol^{-1})	196 x 10^{-20}	196 x 10^{-20}
Specific surface area S (m^2 g^{-1} TS)	169	157

Additional the mean pore radius of aerobic granula was calculated using Equation (4). The wet density D_W of granular sludge was determined in section 5.3.1.3. Combined with the estimated granula porosity ε (section 5.3.1.4), the mean pore radius could be calculated. Assuming that granular sludge cultivated in period 2 and 15 can be structurally compared to the sludge in period 10, a mean pore radius of 1.0-1.1µm could be determined based on the data for specific surface area (Table 9).

5.3.4 Summary

There is not much data about the dye adsorption capacity of activated sludge reported in literature, but the received results are comparable to the existing studies. For activated sludge flocs Smith and Coackley (1984) determined a maximum surface area of 142 m^2 g^{-1} TS. Using similar techniques the specific surface area of biofilm was investigated by Zhang and Bishop (1994a; b). The authors showed results concerning biofilm structure, especially regarding the porosity and mean pore radius based on experimental measurements. Compared to activated sludge flocs a much higher dye adsorption (> 300mmol/kg TSS) was observed, whereas the specific surface area S of the investigated biofilm was in the range 67 to 391 (m^2 g^{-1} TS) and the resulting mean pore radius was 0.3 to 2.8 μm (Zhang and Bishop, 1994a).

Longmuir (1975) found that adsorption of Acid Red AR18 on activated sludge was taking place only under acidic conditions. A pH of 2.5 was found to be suitable for the used dye (Smith and Coackley, 1983), therefore the same pH conditions were chosen for the batch tests in this study. Lowering the pH can affect the sludge structure, hence the observed adsorption capacity may vary at different pH values. For the estimation of surface area and pore radius, structural changes due to pH lowering were assumed to be negligible.

Dye adsorption tests could be successfully applied. Based on batch experiments the maximum specific surface area and the mean pore radius of granular activated sludge were determined. The results indicate that the adsorption properties of flocculent and granular sludge are similar. Based on the assumption that the AR18 adsorption is a monolayer adsorption, by using the results important structural properties of granular sludge could be estimated.

5.3.5 Shear Sensitivity

Kosaric and Blasczyzk (1990) noted that the velocity at the inlet port and the port configuration had a strong influence on granulation of anaerobic sludge. The inlet velocity and the port configuration influence directly the shearing forces. Granulation, either under anaerobic or aerobic conditions, is affected by shearing effects caused by hydrodynamic shear forces (Liu and Tay, 2002).

Further aggregate strength is a crucial parameter since break-up due to hydrodynamics forces causes a shift in particle size distribution and bears the risk of sludge wash-out. Nevertheless there is a lack of quantitative techniques for aggregate strength assessment and effects of agitation are not well understood (Moudgil and Scheiner, 1988; Gregory, 1989). According to investigations of Mikkelsen and Keiding (1999, 2000 submitted) it should be possible to use a standardised batch test to study the strength of flocs, and similar sludge aggregates as granules. In this chapter aggregate strength was quantified in terms of the shear sensitivity (k_{SS}) as a standardised parameter, based on the recent adhesion-erosion (AE) model by Mikkelsen and Keiding (1999) (section 2.2.4). The idea of the AE-model is that activated sludge, consisting of flocs and smaller sized dispersed cells, may be considered as a system of two chemical species. One of the species (single cells) may adhere to the other (flocs). In this model the shear sensitivity quantifies the degree of dispersion for low total solids contents and

intermediate turbulent shear levels. It reflects the affinity of adsorption/desorption of primary particles to flocs or granules. By this the interaction energy between sludge colloids can be characterised (Mikkelsen and Nielsen, 2001).

Break-up experiments were performed in order to investigate the shear sensitivity in relation to the sludge content (section 4.7.4). The shear tests were performed by the procedure described by Mikkelsen and Keiding (1999). Dispersed mass concentrations were estimated by use of the turbidity to mass concentration conversion factor of 1.2 mg SS/l/FTU reported by Wahlberg (1992). The dispersed mass concentration at equilibrium was estimated by polynom fitting.

The batch experiments showed that the turbidity increased rapidly when granular sludge was exposed to turbulent shear. For long shearing times, the desorption rate levels off. The turbidity seems to be at an equilibrium level. The curves further indicate that turbidity increases with increasing sludge concentration (Figure 29). Further the initial turbidity seemed to increase with increasing sludge concentrations, which is in good agreement with sludge floc investigations of Mikkelsen (1999) and Mikkelsen and Nielsen (2001).

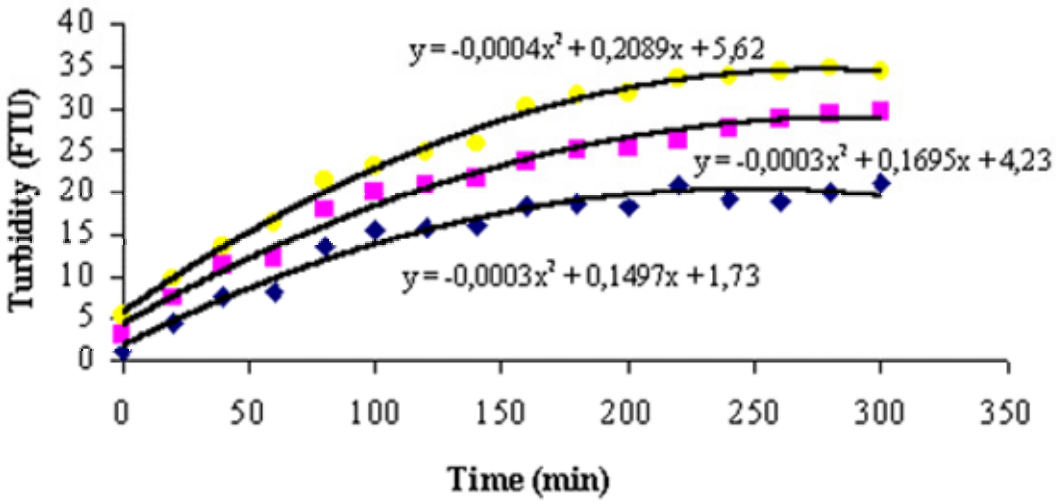


Figure 29. Turbidity vs. shearing time for granular activated sludge at G 0 800 s^{-1}. Solid concentration (◆)1.8; (■) 2.45; (●) 3.1 g SS l^{-1}.

Based on a standardised shear test experiment with a root-mean-square velocity gradient G = 800 s^{-1} and low solid contents (SS < 4 g l^{-1}) a simplified equation (6) can be used to estimate the shear sensitivity k_{SS} (Mikkelsen, 2001):

$$k_{SS} = m_{d,\infty} / m_T \qquad (6)$$

where m_T is the total solids concentration and $m_{d,\infty}$ is the equilibrium dispersed mass concentration (colloid content). By using the turbidity measurements at different sludge concentrations (1.8; 2.45 and 3.1 g SS l^{-1}) k_{SS} values in the range 0.012 to 0.014 with an average of 0.013 could be measured. Mikkelsen (1999) reported for activated sludge flocs an

averaged shear sensitivity k_{SS} of 0.062 ± 0.049. The results of this study indicate a lower shear sensitivity of granular sludge, cultivated in laboratory SBR, compared to activated sludge flocs. Statistically more measurements are necessary to ensure the indicated trend.

Investigations were carried out to get correlation's between granula structure, composition and dewatering properties of the granular sludge.

5.3.6 Discussion and Summary

Separation efficiency is largely affected by the activated sludge structure represented by parameters like particle size distribution, shape, density and porosity of the particles (Moudgil and Scheiner, 1988; Waite *et al*., 1998) or especially by the fraction of small (micron sized) particles (Akers and Machin, 1979; Tomi and Bagster, 1978; Mikkelsen *et al*., 1996).

Mikkelsen and Keiding (1999) described that although the dispersed primary particles only contribute with a small fraction of the total sludge mass, there are indications that they may have a significant impact on solid-liquid separation. Supernatant turbidity has been reported to correlate directly with the free bacteria numbers (Sorensen et al., 1995), which confirms smallest particles being the poorest settlers. Primary particles have been mentioned to have a considerable effect on specific cake resistance (Wu et al., 1985; Rasmussen et al., 1994). Thus the extent of primary particle incorporation into flocs seems to have a significant impact on both clarification (turbidity) and sludge dewatering. Therefore, the relative magnitude of the two size classes, i.e. primary particle mass to total sludge mass may be an important quantity for solid-liquid separation (Mikkelsen, 1999).

The method, based on a standardised break-up experiment, to characterise shear sensitivity was successfully used. The experimental observation that granular sludge has a relatively low shear sensitivity were confirmed by the batch-tests.

Regarding sludge dewatering, it has often been observed, that the presence of dispersed particles has a negative effect on the filtration rates (Mikkelsen et al., 1996; Rasmussen *et al*., 1994; Lawler *et al*., 1986). The increased resistance is believed to be due to blinding of the filter medium with small particles (Sorensen *et al*., 1995). Hence the realised good filtration properties of granular sludge might be explained by the low number of dispersed particles. Since the smallest particles are critical to the filtration rate, it is likely that aggregate strength should be characterised with respect to release of small particles from sludge aggregates, rather than aggregate fragmentation.

5.4 Diffusion and Mass Transfer

5.4.1 Microsensor Measurements

Microscale analyses of chemical species have in general high spatial resolution, rapid response and minimal disturbance of the analysed substrate. The minimal disturbance of the samples does not mean, however, that no disturbance occurs. Although microsensor tips may be thin, introduction of the sensor to deeper layers may lead to considerable disturbance from microsensor shaft. In such a case the recording of multiple concentration profiles should not be performed at the same location. Concentrations profiles in this study were all performed at different locations at one single granula or performed at different granules.

Furthermore even very slim electrodes with tip diameters of a few micrometers cause changes in the thickness of diffusion boundary layer, corresponding to an apparent higher flow rate when inserted from above (Kühl and Revsbech, 2000).

Diffusion is considered to be the main transport mechanism regarding biofilm and also important for suspended sludge systems. Several studies have been attempted to determine diffusion coefficient in activated sludge or biofilms (e.g.: Fan *et al.*, 1990; Fu, 1993; Fu *et al.*, 1994; Zhang and Bishop, 1994a; b). Data on the diffusion of oxygen into sludge aggregates should be obtained. Whereas the hydrodynamic situation and nutrient concentration are determining factors for mass transfer investigations and the thickness of the diffusion boundary layer is directly related to the hydrodynamic environment.

Therefore a set of oxygen microprofiles in relation to COD and oxygen level were recorded for different granula, totally 40 with a diameter range of 3.6-4.0 mm. The micro-profiles were determined within single granula, harvested from the reactor Additional liquid-ion-exchanging membrane (LIX) microsensors for NH_4^+, NO_3^- and NO_2^- (de Beer *et al.*, 1997) were used to investigate the microenvironment of aerobic granular sludge. To measure chemical gradients single granules were placed to a vertical net-jet flow system (Ploug *et al.*, 1998). An upward flow was applied and aggregates floated just above the netting. For the determination of oxygen a microoptode-system (Klimant *et al.*, 1995) was used (section 2.8.2 and 4.5).

Granular sludge samples were mainly taken in phase 10 (section 4.2, Table 2) eight weeks after granulation. The sequencing batch reactors were in steady-state regarding nutrient removal. Dissolved oxygen (DO) penetration into granula was studied in relation to different substrate conditions. The maximum oxygen penetration depth decreased with increasing substrate availability (Table10, Figure 30). Compared to the high COD level as characterised in Table10 (condition A) oxygen could penetrate significantly (160 up to 1320 μm) deeper into the aggregate in conditions B-D. Granula in general were only partially penetrated by oxygen. By using a buffered micro-nutrient solution without adding organic carbon (Table10,

set D) oxygen could penetrate up to the inner core of the sludge aggregate. Whereas for substrate rich conditions A-C anoxic or anaerobic microniches were present within granula.

Figure 30 shows representative dissolved oxygen gradients for granular sludge with a maximum diameter of 4.0 mm. Dissolved oxygen concentration in the bulk water was in the range of 5.7-5.9 mg l^{-1}, which is similar to the DO at the end of the experimental SBR cycle (see Figure 7, section 5.1.1). At the granula surface oxygen concentrations ranged from 3.5-5.0 mg l^{-1} for the various nutrient conditions A-D (Table10). Thus the diffusion boundary layer ranged from 400 to 700 µm, respectively.

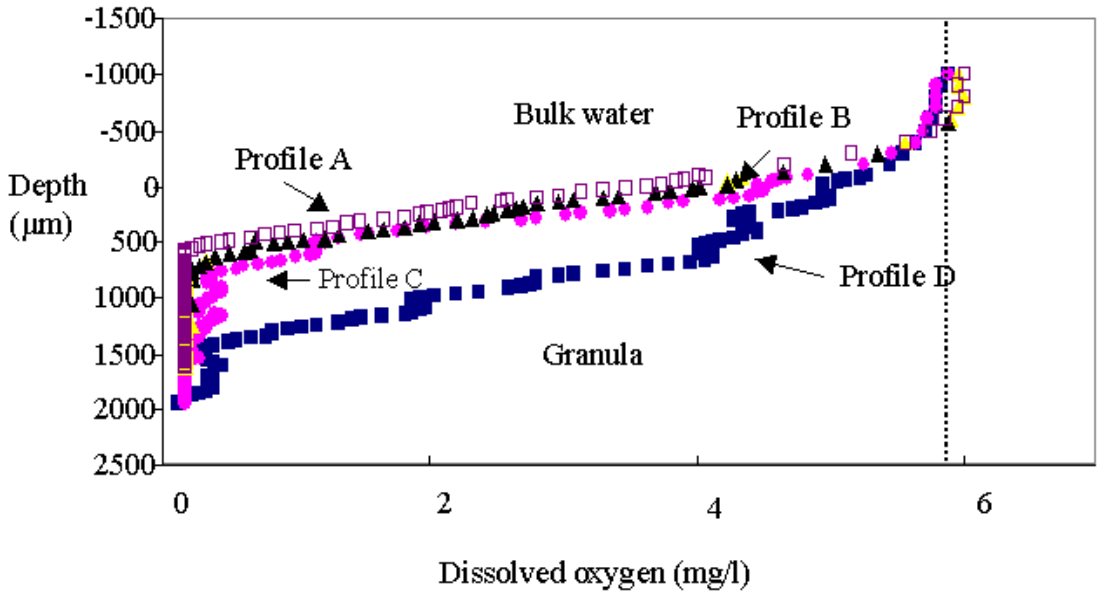


Figure 30. Dissolved Oxygen Profiles within Granula in relation to the Nutrient Situation

Table 10. Dissolved Oxygen Concentrations at Granula Surface and Oxygen Penetration Depth within Granula for different Substrate Conditions (Figure 30)

Profile	Substrate situation	Oxygen level at granula surface (mg l^{-1})	DO ≤ 0.5 mg l^{-1} (µm)	Max. oxygen penetration (µm)
A	Synthetic wastewater 600 mg COD l^{-1}, 30 mg NH4 l^{-1} *	3.5 ± 0.35	460 ± 50	580 ± 50
B	Synthetic wastewater 400 mg COD l^{-1}, 20 mg NH4 l^{-1} *	3.9 ± 0.25	560 ± 50	740 ± 50
C	Synthetic wastewater 200 mg COD l^{-1}, 10 mg NH4 l^{-1} *	4.5 ± 0.15	730 ± 50	1300 ± 50
D	Buffered micronutrient solution < 10 mg COD l^{-1}, 0 mg NH4 l^{-1} *	5.0 ± 0.1	1360 ± 50	1900 ± 50

* Synthetic wastewater composition: Glucose + peptone, nutrients, micronutrients; pH 7

5.4.2 Reaction Kinetics

Microsensors are increasingly used for biofilm and sludge floc investigations (de Beer *et al.*, 1993a,b; 1994; 1997a,b; 1998; Horn and Hempel, 1995; 1997). This *in situ* techniques allow evaluation of parameters of diffusion-controlled reactions in sludge aggregates. Lewandowski *et al.* (1991) described the determination of reaction kinetics in biofilms. Based on the measurements to determine oxygen gradients the related diffusion coefficient D_g and the half velocity coefficient K_s have been calculated.

The dissolved oxygen concentrations in the bulk water referring to substrate situation C and D (Table 10) decreased from 5.7 and 5.9 mg l^{-1} to zero in the granule centre (Figure 30). Dissolved oxygen concentrations at the aggregate-water interface were determined to be 4.5 and 5.0 mg l^{-1}. Below DO concentrations of 0.5 mg l^{-1} the slope of oxygen gradients changed significantly, therefore this concentration was used as the lower limit (C_0). Locating the position of the aggregate surface at the dissolved oxygen profile permitted separation into two parts, on the one hand in the water and on the other hand in the granule itself. The mass transfer boundary layer thickness was 500 ± 50 µm and the decrease in dissolved oxygen concentration was 1.2 and 0.8 mg l^{-1} for condition C and D. Inside the granula the diffusion layer was 700 µm (C) and 1400 µm (D), respectively (Figure 30). The related decrease in dissolved oxygen concentration within this layers was 4.0 (C) and 4.5 mg l^{-1} (D).

5.4.3 Theoretical Considerations

The decrease rate of substrate concentration in the aggregate can be described by an equation resulting from a differential mass balance:

$$\left(\frac{\delta C}{\delta t}\right)_g = D_g\left(\frac{\delta^2 C}{\delta x^2}\right)_g - \frac{V_{\max} * C}{K_S + C} \qquad (7)$$

where D_g is the diffusion coefficient for the dissolved oxygen in the granule ($cm^2\ s^{-1}$); C is the dissolved oxygen concentration at a point x (mg l^{-1}); V_{max} (mg l^{-1} s^{-1}) is the maximum reaction velocity and K_s (mg l^{-1}) is the half-velocity coefficient.

The steady-state concentration within a biofilm $(dC/dt = 0)_f$ is achieved when consumption equals the rate of transport due to diffusion:

$$0 = D_g\left(\frac{\delta^2 C}{\delta x^2}\right)_g - \frac{V_{\max} * C}{K_S + C} \qquad (8)$$

Equation (8) cannot be solved exactly but it can be derived once (Lewandowski *et al.*, 1991). Using this technique the integration constant can be found from the boundary conditions. If

the granule is not totally penetrated, then $(dC/dx)_g = 0$ for C = 0 (no mass transport beyond this depth).

Thus, for a partially penetrated aggregate,

$$\left(\frac{\delta C}{\delta x}\right) = \sqrt{2\frac{V_{\max}}{D_g}\left(C - K_S * \ln\frac{K_S + C}{K_S}\right)} \quad (9)$$

The reaction rate (R) is equal to the rate of mass transfer across the granule-water interface and can be directly calculated from the above equations as

$$R = A * D_g\left(\frac{\delta C}{\delta x}\right)_g \quad (10)$$

where A is the area of the granule-water interface.

The reaction rate at the granule-water interface can be described as

$$R = A\sqrt{2V_{\max} D_g\left(C_g - C_0 - K_S * \ln\frac{K_S + C_g}{K_S + C_0}\right)} \quad (11)$$

where C_g is the granule-water-interface substrate concentration and A is the granula surface area (cm^2).

5.4.4 Half-saturation Coefficient K_S

The growth of heterotrophic bacteria on readily degradable substrates can be described by the maximum specific growth rate and the half-velocity coefficient K_S. These parameters are very dependent on the nature of the wastewater and biomass grown in different reactor configurations exhibits different values of K_S even though the reactors were operated at the same loading. Thus large ranges of values have been reported in the literature and care must be used in the collection and interpretation of kinetic data (Henze *et al.*, 1986).The concentration profiles (Figure 30) can be divided into two parts: that in the bulk water and that in the aggregate. The aggregate diameter was 4.0 mm, hence the diffusion pathway inside the aggregate was 2.0 mm. Third order polynomial curve-fitting procedure was used to estimate the observed dissolved oxygen profiles (Figure 31, 32).

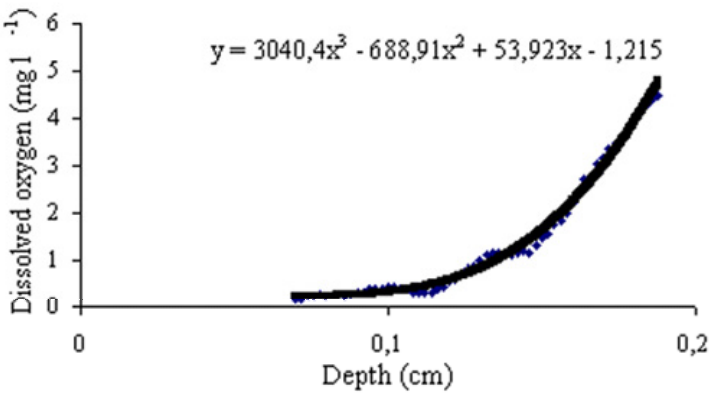


Figure 31. The Dissolved Oxygen Profile within Granula and Polynomial Regression Curve for *in situ* Conditions C (Table 10)

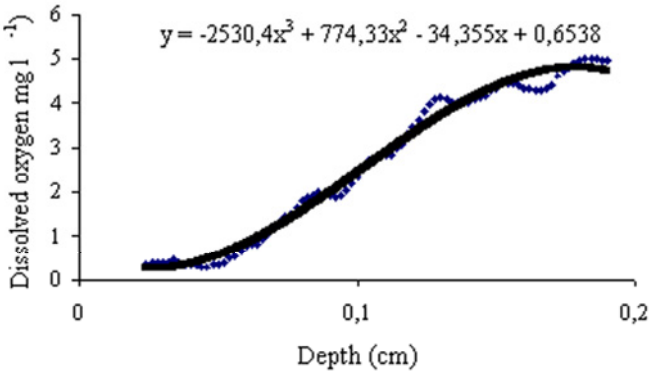


Figure 32. The Dissolved Oxygen Profile within Granula and Polynomial Regression Curve for *in situ* Conditions D (Table 10)

Inversion of equation (8) permits estimation of the K_S value:

$$\left(\frac{\delta^2 C}{\delta x^2}\right)^{-1} = \frac{D_f * K_S}{V_{\max}} \frac{1}{C} + \frac{D_f}{V_{\max}} \qquad (12)$$

The slope divided by the intercept in the plot (Figure 33 and 34) of the inverse of the second derivative against the inverse of the concentration gives K_S. The second derivatives were evaluated based on the oxygen profiles (Figure 31 and 32).

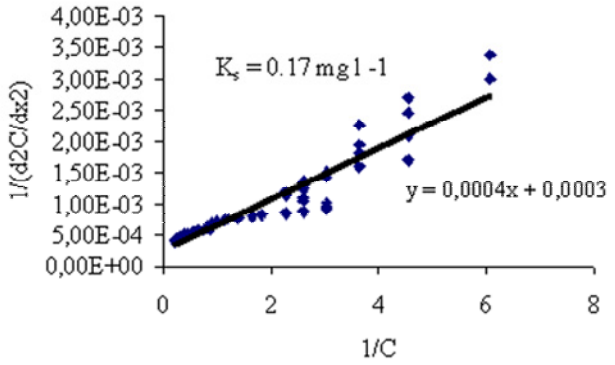


Figure 33. Determination of the Half Saturation Coefficient by Linear Regression [based on Equation (12)] for *in situ* Conditions C (Table 10)

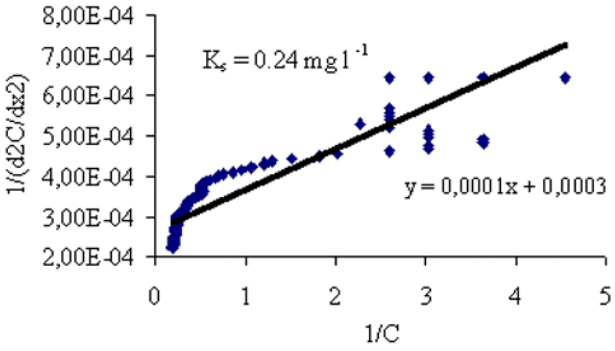


Figure 34. Determination of the Half Saturation Coefficient by Linear Regression [based on Equ. (12)] for *in situ* Conditions D (Table 10)

For the different *in situ* conditions C and D, K_s values of 0.17 and 0.24 mg l^{-1}, respectively were determined.

Wastewater treatment plants generally work at very low actual S_0/X_0 ratios (Brouwer et al, 1998). Therefore S_0/X_0 ratio can be crucial for the estimation of biokinetic parameters (Spanjers and Vanrolleghem, 1995). The applied S_0/X_0 ratio in the described experiments can be considered low (approx. 0.5). The determined values for K_S are in agreement with reported data (Table 11), but higher K_S values can be found. This could be explained by different experimental conditions in various studies concerning respirometric batch experiments. In some investigations high S_0/X_0 ratios were used and this can result in a considerable population shift among the fast-growing organisms (Grady *et al.*, 1996).

A direct comparison of the K_S values in this study and the K_S values presented in Table 11 is limited, because of methodical differences of the determination procedure. However, all K_S values are determined by respirometric measurements.

Table 11. Different K_S Values estimated by Respirometric Measurements

Authors	Year	C-oxidation K_S (mg l^{-1} COD)	S_0/X_0 ratio (g COD g COD^{-1})
Chudoba *et al.* (b,20)	1989	0.4-3.2	-
Slide and Dare (a,20)	1993	1.5-3	-
Spanjers and Vanrolleghem (a, 20)	1995	0.2-4.7	0.05
Kong *et al.* (b, 25)	1996	0.4-1.2	-
Brouwer *et al.* (a, 20)	1998	0.2-1.0	0.06-0.1
This study (b, 20)	2001	0.17/0.24	(0.05)

Notes: (1) Measured with (a) wastewater, (b) biodegradable organic compounds; (2) Measurement temperature at 20 to 21°C (20) or 25°C (25).

5.4.5 Diffusion Coefficient

Based on the dissolved oxygen microgradients (Figure 30) the diffusion coefficient of dissolved oxygen can be calculated. The flux of oxygen through the granule-water interface is:

$$J_g = D_g \left(\frac{dC}{dx} \right)_g \tag{13}$$

Where subscript "g" stands for granula. The flux of oxygen through the diffusion layer is:

$$J_w = D_w \left(\frac{dC}{dx} \right)_w \tag{14}$$

Where subscript "w" stands for water. The flux continuity must be preserved at the water-granule interface ($J_g = J_w$).

$$D_g = D_w \frac{\left(\frac{dC}{dx} \right)_w}{\left(\frac{dC}{dx} \right)_g} \tag{15}$$

The derivatives $(dC/dx)_g$ in the granule and $(dC/dx)_w$ in the water phase can be determined graphically from the slopes in Figure 30. The diffusion coefficient of dissolved oxygen different at the granula-water interface was calculated from equation (15). The results for different *substrate* conditions, referring to Table 10 are summarised in Table 12.

For the dissolved oxygen diffusion coefficient in water, the value $D_w = 2.0 \times 10^{-5}$ cm^2 s^{-1} (21 °C) was used (Lewandowski *et al.*, 1991).

Table 12. Determination of the Dissolved Oxygen Diffusion Coefficient at the Granula-Water Interface

Substrate conditions (referring to Table 10)	A	B	C	D
Concentration gradient in water $(dC/dx)_w$ (mg l^{-1} cm^{-1})	35.4	31.7	24.0	16.0
Concentration gradient in granula $(dC/dx)_g$ (mg l^{-1} cm^{-1})	65.2	60.7	57.1	32.1
Diffusion coefficient ratio D_g / D_w	0.54 ± 0.08	0.52 ± 0.07	0.42 ± 0.05	0.5 ± 0.06

Based on the DO concentration profiles and theoretical models DO flux and diffusion coefficient in microbial granula were estimated. The ratio of the diffusion coefficient in granula in relation to the diffusion coefficient in water ranged from 0.42 to 0.54 with an average of 0.5. This average equals an absolute value of the diffusion coefficient in granula of $1.0 \pm 0.12 \times 10^{-5}$ cm^2 s^{-1}. The dissolved oxygen flux through the water-granula interface is in the range of $J_{w,\,min} = 3.2 \times 10^{-7}$ to $J_{w,\,max.} = 7.1 \times 10^{-7}$ mg s^{-1} cm^{-2}.

A model describing biofilm respiration rates [eq. (11) adopted from Lewandowski *et al.*, 1991] was used to describe aggregate activity as a function of oxygen concentration at the aggregate-water interface. The bulk oxygen concentration and bulk hydraulic conditions influence the granula respiration rate only through the shape of the dissolved oxygen profile. All parameters of the model were experimentally accessible. Similar to the biofilm investigation calculated parameters are site specific. Therefore the data only apply to the specific location in the aggregate penetrated.

5.4.6 Anoxic and Anaerobic Microniches

The occurence of anoxic microniches in aerated activated sludge has been investigated in the past by different methods (Schramm *et al*., 1999). One preferential tool are microsensors. A combined approach of microsensor analysis and molecular techniques (chapter 5.2) was used for the experiments to obtain a better understanding of nitrifying and denitrifying bacteria in aerobic granula. Ion-selective microsensors for ammonium and nitrate have been developed and applied to nitrifying aggregates (de Beer *et al.*, 1993a).

Hence microsensor measurements, to determine microgradients of oxygen (Figure 30), ammonium-, nitrate- and nitrite-ions, were used in single granula harvested from the reactor. As described in section 4.5.3 single granules were placed to a vertical net-jet flow system (Ploug *et al.*, 1998). Using LIX microsensors (section 4.5.2) nitrite could not be detected within granular sludge, hence the gradients refer to ammonium and nitrate (Figure 35).

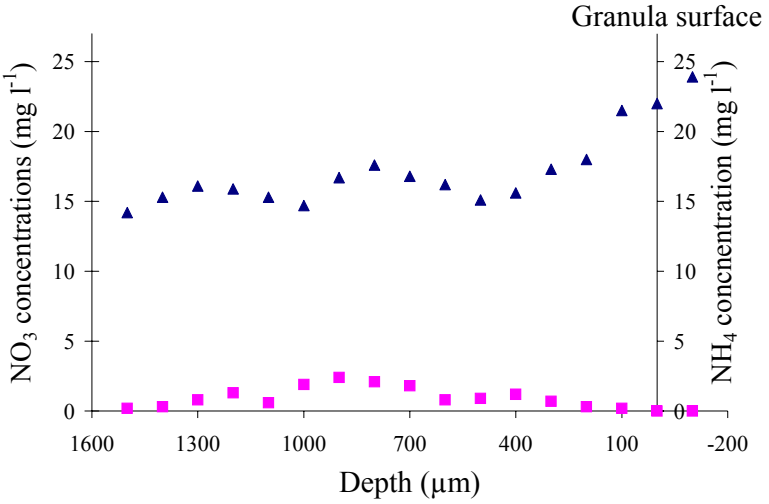


Figure 35. Microprofiles of Nitrate (■) and Ammonium (▲) in Granula (Diameter 3.2 mm) Referring to the *in-situ* Conditions B (Table 10; NH_4-concentration 26 mg l^{-1})

Oxygen, ammonium and nitrate could not be detected simultaneously for technical reason. Microsensor penetration of the same granula was limited by the fact that every measurement disturbed the aggregate structure. Therefore several granules of the same size class and sampling were used to study microgradients of different chemical species. Granula with a diameter range of 2.5 to 4.5 mm were studied. The DO concentration in the flow cell was about 5.5 mg l^{-1} and oxygen diffusion can assumed to be similar as presented in Figure 30. Figure 35 shows that ammonium concentrations decrease up to the centre of the granule. The recorded profiles identified nitrate concentrations up to 3 mg l^{-1} in the core of granules. A significant concentration increase was detectable for depth of 400 to 1500 µm. It can be concluded that anoxic zones in granula were present. The substrate and DO level were comparable to the SBR process conditions, thus simultaneous nitrification/denitrification can be expected to occur during aerated periods.

5.4.7 Indications for Advective Transport

There are studies indicating the possibility of advective transport in activated sludge and biofilm systems. Schramm *et al.* (1998a) suggested advective transport through the porous structure of activated sludge flocs to be responsible for irregular oxygen gradients. Similar reports exist for biofilm investigations (De Beer *et al.*, 1994). Lee *et al.* (1996) investigated the settling of sludge flocs and supposed flow through flocs, too.

Oxygen microprofiles of aerobic granula with various diameters and in different experimental phases (Table 2, phase 7, 10, 14) were recorded. The exemplary oxygen microprofile in 36 indicates the existence of advective flow within the granular structure. Profile I is typical for most measurements and shows more or less regular, hence diffusional transport through the extracellular polymeric matrix of granular sludge. Whereas profile II, which is recorded with granula of the same experimental phase and size class, has an irregular shape and oxygen is probably transported to the aggregate core through cores or channels, therefore oxygen is available at deeper layers.

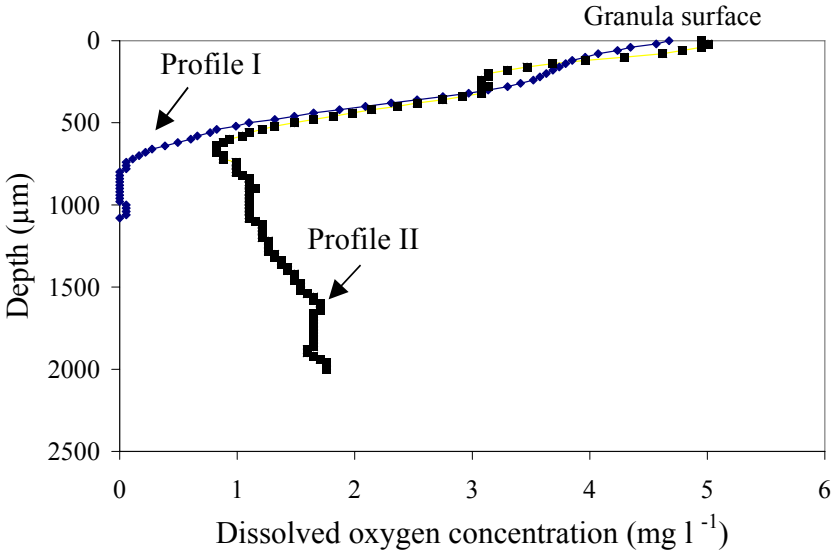


Figure 36. Dissolved Oxygen Microprofiles Detecting Pores and Channels (Profile II). Granula Diameter 3.5 mm

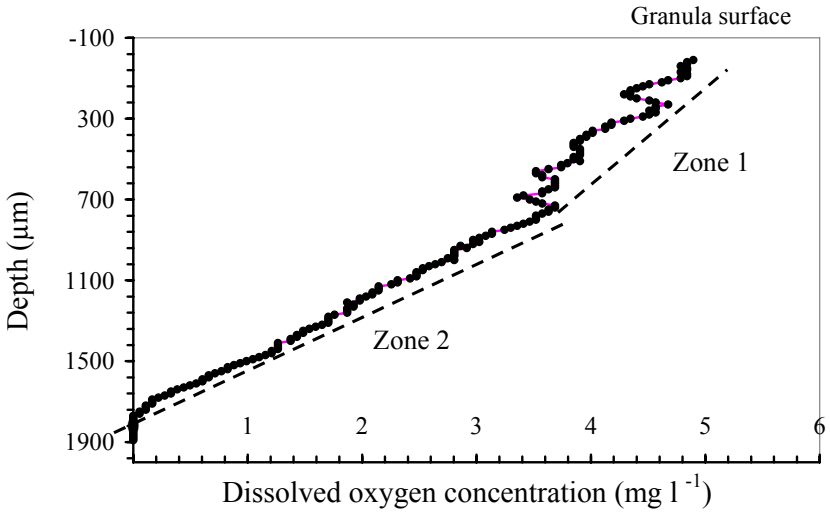


Figure 37. Different Zones within Filamentous Aerobic Granula (Phase 7, Table 2)

In the very first days after granules formed, their surface was often characterised by filamentous microorganisms. Due to the irregular surface structure of the upper aggregate zones (zone 1, Figure 37) flow through this zones could be possible. By this an advective transport additional to the dominating diffusion could be explained. Figure 37 further shows that in the deeper layers (zone 2), the oxygen gradient is regular and hence mass transport is expected to be purely diffusional. The very beginning of the granular surface was difficult to observe and therefore a measurement error of ± 100 µm exists.

5.4.8 Discussion and Summary

Oxygen, ammonium and nitrate concentration profiles could be successfully measured by employing the microelectrode technique. The DO profiles give an idea about the mass transfer processes inside single granula. The oxygen distribution was correlated with the mainly homogeneous and dense packed structure. Some channels and voids could be detected in deeper layers, but in general the concentration profiles were regular in shape, which indicated prevalence of diffusional transport. This might be explained by the fact that bacteria in the surface layer were densely packed closing possible channels. Therefore mass transport from the bulk liquid through the aggregate seemed to be dominated by diffusion, although channels enhanced mass transfer by convection through the aggregate (Figure 36).

An exact quantification of the advective transport was not possible, but mass transport due to flow in the upper zones of granules was clearly indicated, especially for granules with a filamentous surface. The oxygen transport by flow could be related to turbulence induced by fluid flow. The DO diffusion coefficient D_g could be calculated. Theoretical considerations originally for reaction kinetics in biofilms (Lewandowski *et al.*, 1991) were adopted and additional to the oxygen flux at the granule-water interface the half-velocity coefficient K_s

was determined for different substrate conditions. The average oxygen diffusion coefficient D_g was $1.0 \pm 0.12 \times 10^{-5}$ cm^2 s^{-1} (equal to 0.5 D_w). Hence the dissolved oxygen flux through the water-granula interface is in the range of $J_{w, min} = 3.2 \times 10^{-7}$ to $J_{w, max.} = 7.1 \times 10^{-7}$ mg s^{-1} cm^{-2}.

Several studies have been attempted to determine diffusion coefficient in activated sludge or biofilms (Matson and Characklis, 1976; Smith and Coackley, 1984; Sun *et al.*, 1989; Fan *et al.*, 1990; Fu, 1993; Fu *et al.*, 1994; Zhang and Bishop, 1994a; b) and showed that the rate limiting step can be mass transfer through the microbial aggregate to the active sites. Matson and Characklis (1976) measured the diffusivity of oxygen through activated sludge by using a diffusion apparatus consisting of two well-mixed chambers and a oxygen permeable membrane in between. In their study oxygen diffusivity varied from 20 to 100% of is value in water and a glucose diffusivity from 30 to 50%. The determined diffusivity for oxygen through activated sludge was 1.27 x 10^{-9} m^2 s^{-1}. Whereas Fu *et al.* (1994) determined an oxygen diffusion coefficient in the range of 0.5 to 2.4 x 10^{-9} m^2 s^{-1}.

When oxygen was the rate limiting factor in granula, increased organic loading caused more consumption of oxygen by heterotrophic bacteria. This can cause an inhibition of nitrification processes, because of oxygen shortage. Most of the oxygen and probably most of the substrate as well was used in the top layers of the granule. If any substrate was available the dissolved oxygen concentration decreased within the upper 20-25% of the total aggregate diameter (400-500 μm) to values in the range of 0.8 to 2.0 mg l^{-1} depending on the substrate concentration (Figure 30). Hence in the top layer, the bacteria might be in an exponential growth phase so that most organisms were metabolically active. Bacteria in deeper layers might therefore be in conditions of insufficient oxygen.

The results indicate that most of the bacteria in sublayers (500 μm and deeper) might be in a stationary growth or in decay phase. This is in accordance with the distribution of active microorganisms in the top layer of granula, as determined by in section 5.2.3. The results showed that the cells at and near the surface of the granules show the highest ribosome content indicating maximum activity at the surface of the granules. Deeper inside the granules the bacteria possess a drastically reduced ribosome content.

Regarding similar biofilm investigations, Zhang *et al.* (1994) stated that competition for substrates is the main reason for the biofilm structure to be stratified. Their results also showed that the bacterial population had a non-uniform spatial distribution. Similarly the oxygen concentration profiles of aerobic granules showed that oxygen is limited to upper layers of the aggregate and therefore growth is limited for aerobic bacteria. As the aggregate diameter increases, for the reason of substrate competition and mass transfer resistance, the sublayers starts to shift from an aerobic to a facultative dominated population.

In deeper layers the microorganisms might be limited by low DO concentrations. The degradation of organic COD might be ineffective. In anoxic or even anaerobic conditions

simultaneous denitrification could be realised. Furthermore the stratified structure of aerobic sludge aggregates including anaerobic niches might give the opportunity to remove substrates, which are not or difficult biodegradable. In granular sludge different anaerobic and aerobic bacterial groups could coexist in close contact and therefore the ability to degrade certain substances might be improved.

Microorganisms inside granules might be dormant. The work of Kaprelyants and Kell (1996) indicated that most bacteria probably do not die, but become dormant. Van Loosdrecht and Henze (1999) concluded based on their literature review that cell death due to lack of substrate probably hardly ever occurs. Furthermore they state that internal decay does not reduce the number of micro-organisms significantly, but it reduces the weight and activity of the biomass. Internal decay takes place in all microbial systems that are exposed to famine conditions. Van Loosdrecht and Henze (1999) mention that there is almost no natural environment with a constant supply of substrate and therefore bacteria in general should have a storage pool of substrate in their cells. In the presence of external substrate, growth and substrate storage occur. In the absence of external substrate the internal substrate is used for growth and maintenance/endogenous processes (van Loosdrecht *et al.* 1997).

5.5 Nutrient Removal

5.5.1 COD Removal

Regarding substrate and inorganic nutrient removal granular sludge showed in general very similar treatment properties compared to flocculent activated sludge. COD removal was the main process parameter to evaluate the operation of the reactors. The organic loading in different experimental periods varied in the range of 0.6 to 3.6 kg $(m^3\ d)^{-1}$). In general a removal of the organic loading of up to 98% could be realized. Cycle times of 4 and 6 hours were used. The typical COD removal curve of granular sludge is shown in Figure 38. Removal efficiencies ranged from 82 to 98%, normally more than 90 to 95%. MLSS was typically in the range of 5 to 6 g l^{-1}, but values of up to 10 g l^{-1} could be measured. Granulation of the activated sludge seemed to slightly improve the removal efficiency. This could be explained by the fact that the microbial population was already adapted to the substrate after two to three weeks.

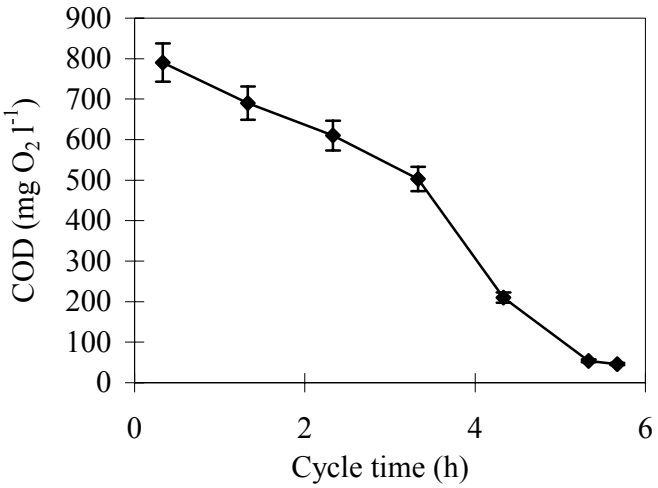


Figure 38. COD Removal of Granular Sludge in the Lab-Scale SBR (Period 9, Table 2)

In the different experimental phases aerobic and alternating anaerobic/aerobic treatment conditions were used to achieve enhanced nutrient removal. COD removal was not affected by the introduction of an anaerobic cycle phase. The substrate uptake in the non-aerated and non-mixed phase (1.55-1.65h) ranged from 25 to 38%, normally around 30% depending on the influent concentration. Main COD reduction could be observed in the aerated phase. Effluent COD values of 30 to 50 were normally observed.

5.5.2 Nitrogen Removal

Biological nitrogen removal in wastewater treatment systems is performed in a combined process of nitrification and denitrification (see section 2.1.1) and carbon removal. There are continuous flow sludge systems using pre- or post-denitrification, but also simultaneous nitrification/denitrification takes place depending on the process sequence and conditions. In any of theses system alternating aerobic (O_2 present as electron acceptor) and anoxic (NO_3^- present as electron acceptor) periods can be identified.

When nitrification occurs, however, the effects of pH have to be taken into consideration. The buffer capacity of the substrate used was sufficient to maintain the pH in a range of 6.8 to 7.5. Nitrification efficiency, in steady-state conditions, was in the range of 90-99% and usually a simultaneous denitrification could be observed (Figure 39). Nitrogen loss could be observed for flocculent sludge, but it did not exceed more than 10%. In comparison with it granular sludge showed nitrogen loss up to 100%. A possibly correlation of the granula diameter to the simultaneous nitrification/denitrification could be observed. For granula diameters up to 2 mm, and DO concentrations of 5.5-6.0 mg l^{-1}, nitrogen loss ranged from 50 to 80% (Zhu *et al.*, 2001). For increasing aggregate diameters nitrogen loss reached up to 100%, hence nitrite and nitrate could not be detected during one cycle. Generally nitrite concentrations were to

low to detect, hence the profiles in Figure 39 refer to ammonium and nitrate concentrations during one SBR cycle.

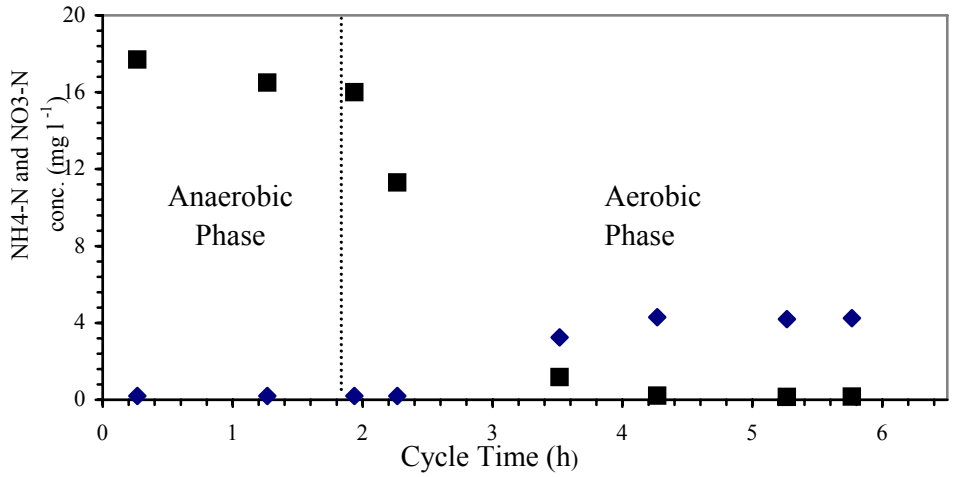


Figure 39. Simultaneous Nitrification/Denitrification during one SBR Cycle. (■) NH_4-N; (◆) NO_3-N

Nitrogen removal is an important aspect of present wastewater processes. Since there exist many nitrogen compounds in typical municipal wastewater and many of the bacteria involved have slow growth rates, the removal process is often hampered (Van Loosdrecht and Jetten, 1998).

Microsensor measurements in this study (section 5.4.1) could detect oxygen limitations within granular sludge. Hence due to the diffusion limitation anaerobic and anoxic zones or pockets existed within the aggregates which might explain the denitrifying activities while aeration periods. But in the present study *in situ* hybridisation techniques in combination with specific oligonucleotide probes were used to directly relate community structure with the morphology and spatial distribution of the detected organisms. Using these modern molecular-biological methods it was not possible to detect ammonia- or nitrite-oxidizing bacteria in granular sludge (section 5.2.2). Therefore inhibition tests were carried out using 2.4-dinitrophenol ($C_6H_4N_2O_5$), a specific inhibitor of biological activity (Bedard and Knowles, 1989), to investigate the nature of the nitrogen loss.

Two batch reactors were filled with 250 ml aerobic granules (2-2.5 mm in diameter), directly sampled from the sequencing batch reactor. According to Bedard and Knowles (1989) to batch reactor (I) 75 mg of the inhibiting agent were added, the second one was used as a control. After 20 min. of stirring ammonium sulfate was added up to a final concentration of 38.0 mg l^{-1} to both reactors. During 4 h of constant, controlled aeration and reaction time 99% of NH_4-N in the control reactor were removed. In contrast the decrease of NH_4-N in the other reactor (batch I) with 2.4-dinitrophenol was stopped at 23 mg l^{-1} (Figure 40). No significant concentrations of nitrite or nitrate could be detected during the batch experiment in both batch reactors.

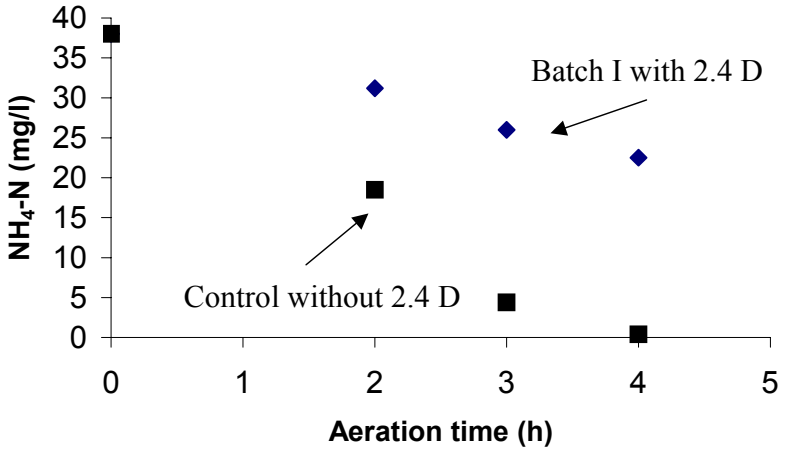


Figure 40. Batch Experiment using 2.4-Dinitrophenol (2.4 D) as Inhibitor (TSS = 5.6 g/l)

The batch results suggest that the majority of the incoming ammonium is removed due to biological activity. No autotrophic ammonia oxidisers could be detected by *in situ* hybridisation, however, possibly heterotrophic nitrifiers can be responsible for oxidation of NH_4-N to nitrate in the system.

5.5.3 Phosphorus Removal

There have been reports claiming that the enhanced biological phosphorus removal (EBPR) could not be accomplished with glucose feed as a carbon source due to the dominating growth of so-called "G-bacteria" which do not accumulate polyphosphate (Jeon and Park, 2000). Despite several studies to investigate EBPR the exact mechanism is not completely understood. To induce the EBPR activity, alternating anaerobic and aerobic or anoxic conditions are required. In general, microorganisms responsible for EBPR, known as polyphosphate accumulating organisms (PAO), are capable of storing organic compounds. Usually short chain volatile fatty acids, poly-3-hydroxy alkanoic acids (PHA), and glycogen are built as internal storage compounds. Under aerobic and famine conditions the bacteria will use the stored compounds to produce energy for cell growth and maintenance (Van Loosdrecht and Heijnen, 1997; Van Lossdrecht and Henze, 1999).

In this study EBPR metabolism was not studied in detail, but in the experimental periods 10 to 15 (section 4.2, Table 2) an anaerobic period was introduced to the SBR cycle to induce bio-P activity. Using a synthetic wastewater with glucose and peptone as carbon sources it was generally possible to achieve efficient phosphorus removal. P-removal with a cycle times 6 (Table 2, period 10, 13, 14, 15) and 8 h (Table 2, period 11). The P-removal efficiency ranged from 57-99%, and was normally more than 60%. The stable P-removal efficiency for several weeks reached to 96-99% (Zhu and Wilderer, 2003). The characteristic P-release in anaerbic phase as well as the synthesis of storing compounds were noticed (see chapter 5.6).

In general the sequencing batch reactors and its process parameters were not optimised for an enhanced nutrient removal. Hence the presented results concerning COD, nitrogen and phosphorus removal efficiency are limited to the specific influent and operation conditions. A further improvement of the removal rates might be expected for optimised conditions. All data are collected using readily biodegradable synthetic wastewater and particulate organic material was not investigated. Hence a direct comparison of removal rates is difficult. In Table 13 the typical range of the removal capacity (Table 2, period 13-15) is listed.

Table 13. Removal Capacity of Aerobic Granular Sludge in Lab-Scale SBR

COD-removal (kg COD / m^3 d)	Nitrogen-removal (kg N / m^3 d)	Phosphorus-removal (kg P / m^3 d)
1.1-3.6	0.1-0.12	0.03-0.04

5.5.4 Discussion and Summary

Summarising the substrate removal, it can be stated that aerobic granular sludge showed efficient and stable COD removal combined with the capability for enhanced nutrient removal. Nitrification and simultaneous denitrification as well as EBPR were observed and removal rates are comparable to municipal activated sludge systems. Simultaneous denitrification processes could be related to diffusion limitation in deeper zones of granular sludge (section 5.4). At high substrate concentration (COD >200 mg/l) dissolved oxygen penetrated only the surface layers and therefore anoxic and anaerobic microniches were present. In section 5.3.2 similar adsorption properties compared to activated sludge flocs were observed, hence dissolved substrates might penetrate granular sludge in a similar way as flocculent sludge. However, the substrate removal capacity of granular sludge cannot be predicted for municipal wastewater, since removal data for particulate material is missing.

Although standard textbooks (Henze *et al*., 1997) still report that *Nitrosomonas europaea* and *Nitrobacter* spp. are the ammonium and nitrite oxidizers in WWTPs, the actual picture is far more complex (Wagner and Loy, 2002).

Purkhold *et al.* (2000) published 16S rRNA and *amoA* sequence data from all recognised ammonia-oxidising bacteria (AOB) and extended the currently used molecular classification schemes for AOB. In the past 16 AOB species could be described by enrichment and isolation. Further investigations using DNA-DNA hybridisation provided evidence for at least 15 additional species. In addition a summary of hybridisation probes for beta-subclass AOB was presented, indicating that the probe Nso 1225 is specific for ß-AOB and has a good sensitivity regarding *Nitrospira* and *Nitrosomonas communis* cluster.

Although the used oligonucleotide probe Nso1225 is generally suitable for the detection of ß-AOB (Purkhold *et al.*, 2000) no positive signals could be identified. In single cases nitrification by heterotrophic bacteria was reported (Kilham, 1986; Van Loosdrecht and Jetten, 1995). Further investigations are necessary to answer the question whether heterotrophic nitrification occurred, insufficient sensitivity of the used gene probe or methodical failures (see chapter 5.2.4) were responsible for the negative analysis.

Still it is not clear which microorganisms are important *in situ* denitrifiers in WWTPs (Wagner and Loy, 2002). Denitrification in anaerobic and anoxic zones within the granules might be catalysed by the *Zoogloea ramigera*-like bacteria (Unz, 1989). Several microbial processes have been reported regarding nitrogen removal in WWTPs by aerobic denitrification and heterotrophic nitrification (Robertson, 1988; Robertson and Kuenen, 1990; Muller *et al.*, 1995; Helmer and Kunst, 1998).

5.6 Intracellular Storage Compounds and Oxygen Uptake

5.6.1 Intracellular Storage Compounds

Storage of PHB, and possibly of other storage compounds, occurs when the substrate uptake exceeds the conversion capacity of assimilatory processes. The amount of substrate which is not needed directly for growth processes, will be used for PHB or glycogen synthesis (Beun *et al.*, 2001b). According to the hypothesis of van Lossdrecht and Heijnen (1997) it may be assumed that regular periods of feast and famine conditions will enrich microorganisms that are capable of balancing their growth independent of the external substrate concentration. The selected bacteria would have a competitive advantage by keeping all cell systems viable even when the external substrate is missing. Therefore two important storage compounds, glycogen and PHB, were investigated for steady-state conditions in period 10 (section 4.2, Table 2).

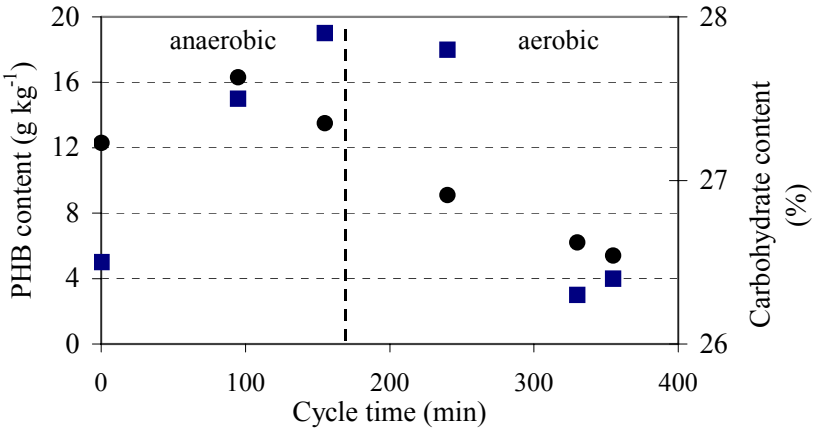


Figure 41. PHB and Total Carbohydrate Content during one Cycle in Period 10: (●) PHB; (■) Carbohydrates

The first sludge samples were taken directly after filling at t = 15 min and the final samples were taken at the cycle end (t = 355 min). The process can be characterised by feast and famine conditions, which are assumed to correlate with the dissolved oxygen concentration in the reactor (section 5.1.1, Figure 7). The feast period should start right after the filling phase, and after substrate removal has been accomplished, approximately after 5 hours for the experimental period 10. Famine conditions preveil towards the end of the SBR cycle.

In Figure 41 the increase and decrease of storing compounds (PHB and glycogen) during one SBR cycle is documented. Glycogen was not measured directly, but the total carbohydrates were determined. The synthesis of carbohydrates in one cycle is assumed to be directly related to the production of glycogen. In the initial anaerobic phase PHB and glycogen are accumulated and both are used for cell growth or maintenance processes in the aerobic phase. Hence it can be concluded that bacteria are present which are capable of storing compounds, probably related to the EBPR process. Hence the cyclic process resulted in a microbial population that used storing compounds to balance their growth independent of external substrates.

5.6.2 Maximum Oxygen Uptake Rate OUR $_{A,max}$

Since oxygen consumption is directly associated with both biomass growth and substrate removal respirometry is a useful technique for modelling and operating the activated sludge process (Spanjers *et al.*, 1998).

The exogenous oxygen uptake rate curve reflects the kinetics of aerobic biodegradation of C and N substrates by heterotrophic and autotrophic microorganisms of the activated sludge. Many authors have reported on slightly different systems for measuring OUR (among others: Vanrolleghem *et al.*, 1999; Kong *et al.*, 1996; Nowak *et al.*, 1994). All the systems have in common that a previously aerated activated sludge sample is continuously mixed in an airtight reaction chamber without oxygen supply. The gradient of dissolved oxygen concentration in this chamber represents the respiration rate of oxygen uptake rate.

In order to determine OUR $_{A,max}$ with such a system two measurements of respiration rates have to be carried out. The maximum autotrophic respiration is measured after the addition of an ammonia salt to a sample of well aerated activated sludge. The results of this measurement includes the endogenous heterotrophic oxygen uptake rate OUR $_{H,e}$. Hence OUR $_{H,e}$ has to be determined separately in a second measurement with the addition of allythiourea (ATU) to inhibit nitrification. Finally, the maximum autotrophic oxygen uptake rate OUR $_{A,max}$ is calculated by subtracting OUR $_{H,e}$ from the previously measured respiration rate (Nowak *et al.*, 1994).

To determine the maximum heterotrophic oxygen uptake rate a defined substrate solution (pH 7.3) was added after previous aeration. To inhibit nitrification ATU was added to a concentration of 13 mg l^{-1}. Respiration was measured in a closed measuring batch reactor (section 4.7). Sludge samples were taken in steady-state conditions of period 7 (section 4.2, Table 2). The observed oxygen uptake rates ranged from 78 to 108 g O_2/(kg oTS h). The maximum autotrophic respiration rate was typically in the range 25-29% in relation to the maximum heterotrophic oxygen uptake rate.

5.6.3 Storage Effects on the Heterotrophic Oxygen Uptake Rate OUR_H

To investigate the effect of storage under different conditions oxygen uptake rates were determined. Aerobic granular sludge was taken from the SBR (Table 2, period 13), washed (section 4.8) and after addition of mineral salts stored under three different conditions (Table 14).

Table 14. Sludge Storage Conditions

	Storage conditions
Sludge sample B	Room temperature (19-21°C), every 4 hours aerated for 15 min
Sludge sample C	Cooled temperature (6-7 °C), not aerated
Sludge sample D	Room temperature (19-21°C), continuously aerated

The endogenous heterotrophic respiration rate $OUR_{H,end}$ (Figure 42) as well as the exogenous oxygen response after substrate addition $OUR_{H,ex}$ (Figure 43) were determined before and after 7 days of storage. In Table 15 the oxygen uptake rates for the different storage conditions and the initial value for endogenous and exogenous oxygen uptake rates are summarised. Additional the ratios of the initial OUR to the final OUR after 7 days of storing are listed in Table 15.

The oxygen uptake without external substrate available before (profile A) and after storing for 7 days (Profile B-D) is shown in Figure 42. The endogenous heterotrophic respiration rate decreased significantly after storing anaerobically at 6-7°C, but also aeration for 7 days lowered clearly the $OUR_{H,end}$. Despite storing under intermittently aeration reduced the endogenous oxygen uptake only 20% in relation to the initial value (Table 15).

After the addition of 10 ml substrate (section 4.7), the oxygen uptake of granular sludge was measured to determine the $OUR_{H,ex}$ (Figure 43). Storing under cooled, anaerobic and storing under permanently aerated conditions decreased the oxygen uptake rate to 43% and 31%, respectively in relation to the initial value. But the sludge viability was slightly affected by storing at intermittently aeration and the $OUR_{H,ex}$ decreased 11% in relation to the initial value (Table 15).

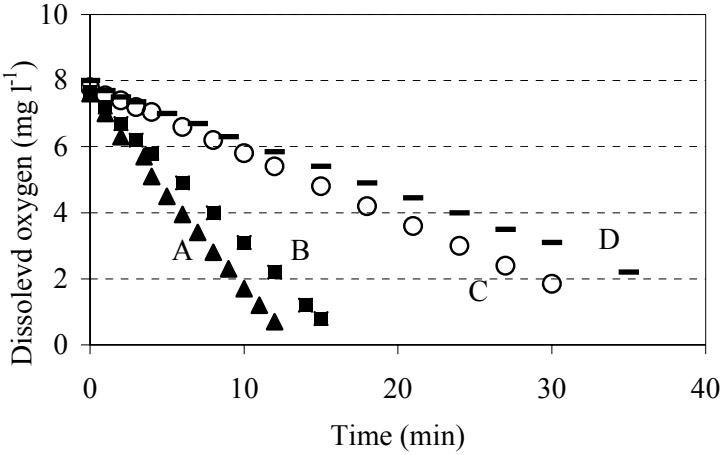


Figure 42. Determination of Endogenous Respiration Rate before (profile A) and after Storing for 7 Days (Profile B-D) referring to Storing Conditions in Table 14

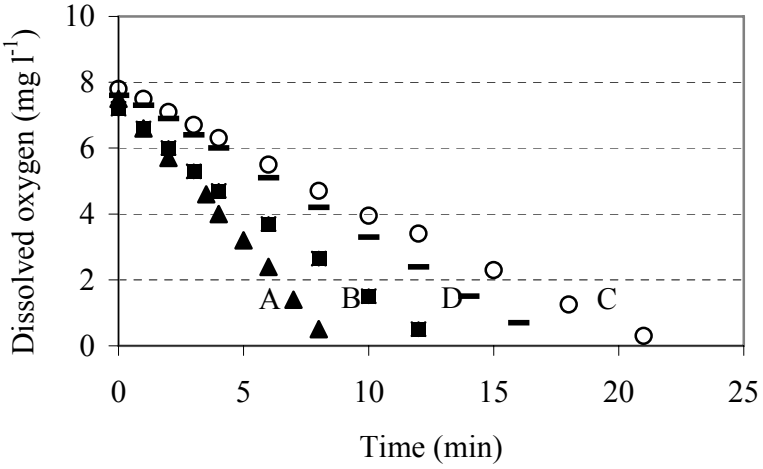


Figure 43. Determination of Substrate Respiration Rate before (profile A) and after Storing for 7 Days (Profile B-D), referring to Storing Conditions in Table 14

Table 15. Endogenous and Substrate Oxygen Uptake Rates after different Storing

	Day 0 Profile A	Day 7 Profile B	Day 7 Profile C	Day 7 Profile D
$OUR_{H,end}$ (mg O_2/ l min)	0.57	0.46	0.2	0.16
$OUR_{H,end7}/OUR_{H,endO}$	-	0.80	0.35	0.28
$OUR_{H,ex}$ (mg O_2/ l min)	0.63	0.56	0.36	0.43
$OUR_{H,ex7}/OUR_{H,SO}$	-	0.89	0.57	0.69

Figure 42 indicates that the endogenous respiration rate after 7 days of storage decreased 20 to 72% in relation to the initial value, which is summarised in Table 15. Exogenous oxygen

uptake after 7 days decreased 11 to 43%. The significant activity loss during aerobic storage can be explained by the fact that continuous aeration possibly oxidised any storage product and further increased the decay of PAO due to lack of the anaerobic phase. Siegrist *et al.* (1999) assumed a PAO decay rate of 0.12 d^{-1}, hence PAO biomass could be reduced by 50-60% in 7 days, if storage polymers are mineralised.

5.6.4 Discussion and Summary

Heijnen (1994) stated, based on a literature review, that biomass synthesis requires a standard amount of energy dissipation. Yield for aerobic growth is appr. 0.5 g COD/g COD for a wide range of aerobic bacteria and substrates. For waste water processes usually a yield of 0.6-0.7 g COD/g COD, based on respirometric observations, is used. The discrepancy between pure culture observation and mixed culture observations can be explained when a fraction of the substrate is converted into storage polymers.

When organisms are growing under limited substrate conditions they need relatively large amount of substrate uptake enzymes, because the uptake is limited by the substrate concentration. If the substrate concentration suddenly rises this results in a rapid uptake of the substrate. This amount of substrate can not directly be converted by the growth processes in the cell. If this substrate could not be converted to a polymer the whole cell metabolism would run the risk of getting out of balance. When the substrate concentration remains high the organisms can adjust their growth rate.

It can be noted that by storing granular sludge at room temperature and using intermittent aeration, the endogenous and substrate respiration rate are lowered not more than 20%. Hence for 7 days a high activity of the granular sludge could be maintained by using optimised storage conditions.

6 CONCLUSIONS

1. Aerobic granular sludge with excellent settling properties could be cultivated using different readily biodegradable substrates and selected process conditions in laboratory sequencing batch reactors.
2. Process conditions which reproducibly led to granule formation included short settling time and high volumetric exchange ratio in combination with a fast filling phase.
3. Filamentous organisms apparently were closely related to the granulation process and could be identified as a structural backbone during the initial phase of granulation. Fungi/yeast were not dominating granular structure. Excess growth of filamentous microorganisms in granular sludge was not observed. Metabolic and kinetic selectors were successfully used.
4. Using molecular methods it was possible to determine the structurally involved filamentous bacteria, *Sphaerotilus natans*. In steady state conditions most of the population consisted of betaproteobacteria (approx. 89%) and gammaproteobacteria (approx. 5%). The full cycle 16S rDNA approach indicated a relatively limited species diversity.
5. Typical floc forming bacteria (*Zooglea ramigera*) could grow and dominate without destabilising the granula structure. Most likely the dominating *Zooglea ramigera* were responsible for a very compact granula structure.
6. Buoyant density of flocculent and granular activated sludge was in a similar range. Granular settling was related to the aggregate size and the settling velocity of single granules could be described by modified Stokes` law.
7. Granular structure was characterised by a metabolically active surface layer and diffusion limitation in the inner core where bacterial decay took place. Anoxic and anaerobic microniches existed in deeper zones. Channels and voids could be detected in deeper layers.
8. Due to the limited oxygen diffusion in granular sludge simultaneous nitrification and denitrification could be realised. Alternating anaerobic/aerobic conditions induced high and stable EBPR activity. However, no nitrifying bacteria or known PAOs were identified by molecular methods.
9. In comparison to flocculent activated sludge a similar specific surface area and related adsorption properties were determined, whereas shear sensitivity seemed to be lower.
10. Oxygen uptake tests of the granular sludge indicated high activity. The respiration rate could be maintained at high level (> 80% of the initial rate) for seven days although aeration was limited to a minimum and nutrient supply were stopped.

11. Summarising the collected data about the formation, structure and functions of aerobic granular sludge, the initially formulated hypothesis for the granulation process could be concluded.

Summary of the Granulation Process

- Sequencing batch reactors were used to select for activated sludge with improved settling properties. Increased wash-out of slow settling flocs in combination with a high substrate gradient in the cyclic process was followed by the formation of initial granules. Especially *Spaerotilus natans,* a filamentous bacteria, was present.
- Filamentous microorganisms formed a structural backbone for the first aggregates which were characterised by entangled filaments. These granules (300-400 μm) gave "floc-forming" bacteria the chance to settle down and develop under protected conditions. Adsorbed to the floc, these bacteria could not easily be washed out of the system and grazing by protozoa was limited.
- Easy biodegradable substrate was available and therefore filamentous microorganisms as well as floc-forming bacteria could grow. But due to the selective pressure of the cyclic process and the inherent selectors, filamentous organisms were repressed more and more with increasing cycle numbers. This selective process gave other species, especially floc-forming ones (e.g. *Zoogloea. ramigera*), the opportunity to develop.
- In an feast and famine regime the EPS production might be enhanced, and in balance to the bacterial growth, shearing forces formed granulated and spherical sludge, which showed similar metabolic activity compared to flocculent activated sludge.

7 PERSPECTIVE - GRANULAR SLUDGE USED FOR BIOAUGMENTATION

In wastewater treatment and especially in industrial plants one major problem is most prominent. The performance of a plant is often hampered by sludge bulking, foaming and poor settling properties of the activated sludge in the secondary clarifier. In general the treatment systems have a large area requirement for reactors and especially settlers. The loss of functionality within a vital stage of wastewater treatment like nitrification bears economical and ecological risks, hence different ways have been investigated to cope with problems related to poor settling of activated sludge. The basic questions are how to prevent these effects and how to handle them if they occur.

Sludge bulking and foaming is often dealt with chemical settling enhancers, which are cost intensive and burdening the environment. The regain of the desired physiological activity after a breakdown can potentially be realised fast and precisely by bioaugmentation (Van Limbergen *et al*., 1998), however, there are only a few examples for successful bioaugmentation of activated sludge systems (McClure *et al*., 1991; Nußstein *et al*., 1992; Selvaratnam *et al*., 1997). The inoculation of sludge with pure or mixed cultures is often handicapped by the inflexibility of the applied cultures to adapt to the new environment and, as shown in recent investigations (Bouchez *et al*., 2000), by a massive grazing of Protozoa. Hence, single cells were not able to survive long enough to become part of the active community.

As innovative approach granules formed by biological communities could be a reply to some of these problems. For anaerobic processes in comparison to aerobic ones much more compact reactors (e.g. UASB-reactors) have been developed, in which the biomass grows as well settling granules. This makes it possible to accumulate high amounts of active biomass. Although the potential of aerobic granular sludge is not yet widely utilised and full scale applications are missing, information on granules grown under anoxic or anaerobic conditions is available (section 2.7.2) and can be used to draw conclusions.

The presented study indicated that, regarding the possibilities of bioaugmentation, aerobic granules might offer a similar potential as anaerobic sludge. Different bacterial groups in partly anoxic and anaerobic zones were detected in aerobic granules. For anaerobic granular sludge it was already possible to introduce a certain “de novo” degradative pathway by means of inoculation with a pure culture (Ahring *et al*., 1992). This method might be adapted for aerobic granular sludge to develop a protection system for cell cultures during bioaugmentation.

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9 APPENDIX

9.1 Abbreviations

Abbreviations	Description
AOB	Ammonia Oxidizing Bacteria
ASBR	Anaerobic Sequencing Batch Reactor
ATU	Allythiourea
BAS	Biofilm Airlift Suspension Reactor
CLSM	Confocal Laser Scanning Microscopy
COD	Chemical Oxygen Demand
Clta	Clones affiliated to *Tolumonas auensis*
CMFR	Completely Mixed Flow Reactor
DAPI	4',6'-Diamidino-2-Phenylindole Hydrochloride
DGGE	Denaturing Gradient Gel Electrophoresis
DIN	Deutsches Institut für Normung e.V.
DLVO	Theory of Derjaguin and Landau (1941) and Vervey and Overbeek (1948)
DO	Dissolved Oxygen
DNS	Deoxyribonucleic acid
EBPR	Enhanced Biological Phosphorus Process
EPS	Extracellular Polymeric Substances
FISH	Fluorescence-In-Situ-Hybridisation
F/M	Food-To-Microorganisms
FTR	Fill Time Ratio
GAC	Granular Activated Carbon
HRT	Hydraulic Retention Time
LIX	Liquid Ion Exchanger
MAC	Micro-Spheres Adhesion to Cells

MAR	Microautoradiography
MLSS	Mixed Liquor Suspended Solids
OLR	Organic Loading Rate
OTU	Operational Taxonomic Unit
OUR	Oxygen Uptake Rate
PAO	Phosphate Accumulating Organisms
PBS	Phosphate Buffered Solution
PCR	Polymerase Chain Reaction
PFR	Plug-Flow Reactor
PHA	Polyhydroxyalakanoates
PHB	Poly-ß-hydroxybutyrate
RNA	Ribonucleic acid
SBR	Sequencing Batch Reactor
SRT	Solids Retention Time
SS	Suspended Solids
SVI	Sludge Volume Index
UASB	Upflow Anaerobic Sludge Blanket
VER	Volumetric Exchange Ratio
VSS	Volatile Suspended Solids
WWTP	Wastewater Treatment Plant

9.2 List of Symbols

A	area covered by each molecule, $m^2 mol^{-1}$
C	dissolved oxygen concentration
C_D	drag coefficient
D_g	the diffusion coefficient, $cm^2 s^{-1}$
D_W	wet density of the sludge, g cm^{-3}
ε	porosity of sludge aggregate, %
G	root-mean-square velocity gradient, s^{-1}
k	floc permeability, m^2
k_{SS}	shear sensitivity
N	Avogadro's number (6.023×10^{23})
Ω	factor that describes the ratio of the resistance experienced by a granule to that of an equivalent solid sphere
ρ_p	density of the primary particles comprising the floc
ρ_w	density of water
r	mean pore radius, µm
Re	Reynolds number
S	surface area per unit mass of porous media, $m^2 g^{-1}$
S_o/X_o	ratio of substrate to microorganisms
Y	adsorbed dye, mol g^{-1}