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**POPULATION DYNAMICS AND KINETICS OF NITRIFYING
BACTERIA IN MEMBRANE AND CONVENTIONAL ACTIVATED
SLUDGE PLANTS**

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Summary

Introduction

During the last decades, municipal wastewater treatment has developed from only carbon to nutrient removal in order to reduce the eutrophication of coastal waters and to prevent adverse effects of ammonia in receiving waters. As a consequence, the required volume for the biological processes has risen. Furthermore, stringent regulations for sensitive receiving waters additionally necessitate a filtration step after the biological treatment. Conversely, space becomes increasingly a limiting factor particularly in urban areas. At the same time, pressure for water reuse rises due to water shortage in arid regions.

Membrane bioreactors (MBRs) for the treatment of municipal wastewater have attracted increasing attention in recent years. Compared to the conventional activated sludge process (CAS) they have a lower footprint due to the omission of the secondary settling tank, need smaller biological tanks and provide the effluent free of solids. However, mostly higher costs and elevated energy consumption in comparison to CAS are still a drawback of MBR, beside the uncertainty of a new technology for the investor. Nonetheless, decreasing membrane prices due to growing competition and lessened energy consumption by optimized operation of MBR will significantly advance their attraction in the coming years. In addition, tightened discharge regulations and the dealing with new substances e.g. endocrine disrupters may contribute to the extension of MBR. Finally, MBR systems for the treatment of the wastewater from single households or apartment houses with subsequent recycling of the treated water are already commercially available.

First full-scale plants were put into operation in past years, adapting the experiences from conventional activated sludge plants. On the other hand, long-term experiences are still missing, which leads to a certain caution and reluctance of the designing engineers and the operators of wastewater treatment plants (WWTPs) in terms of MBR technology.

Aim of thesis

The aim of this work was to investigate the influence of the membrane separation on the activated sludge properties. This included the study of population dynamics as well as the provision of kinetic information. The new insights should help to understand how the broad knowledge and experience from CAS systems can be transferred to MBR systems. Emphasis was placed on nitrification and the involved microbial populations because of their important role in municipal wastewater treatment.

Methodology

A conventional and two membrane bioreactor pilot plants were run in parallel for more than two years. They were fed with municipal wastewater showing a typical diurnal hydraulic variation. The CAS and the MBRs treated the wastewater corresponding to 60 ($18 \text{ m}^3 \text{ d}^{-1}$) and 2 ($0.56 \text{ m}^3 \text{ d}^{-1}$) population equivalents, respectively. The MBRs were equipped with four submerged hollow fibre modules from Zenon (ZW-10) with a surface area of 0.93 m^2 each and a characteristic pore size of $0.1 \mu\text{m}$. The plants were operated with pre-denitrification and the sludge age was kept at 20 days. The startup of the MBR and CAS was done without inoculation.

The nitrifying population was quantitatively analyzed using Fluorescent *In Situ* Hybridization (FISH). Different batch tests were performed to gain information on the kinetics of nitrification.

Quantification of nitrifying bacteria

Quantification of bacteria using Fluorescence *In Situ* Hybridization (FISH), confocal laser scanning microscopy (CLSM) and image analysis is very time consuming and requires the availability of an expensive microscope. Therefore, a rapid method to quantify nitrifying bacteria in activated sludge using FISH and epifluorescence microscopy was developed. The quantification of the biovolume is based on manual counting of the aggregates formed by nitrifying bacteria and determination of their size. Accordingly, the time needed for one sample was only 5 to 15 minutes, compared to about one hour for the quantification with CLSM and image analysis. The method proved to be suitable for the analysis of population dynamics of nitrifying bacteria in activated sludge.

Population dynamics

The identification of the nitrifying community composition using FISH revealed only minor differences between the CAS and the MBR for both ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Accordingly, both systems exhibited the same maximum nitrification rates. Confocal laser scanning microscopy showed that the aggregates formed by nitrifying bacteria were located mostly in the inner part of the flocs and were overgrown by heterotrophic bacteria. Aggregates built up by NOB were generally smaller than those formed by AOB. Furthermore, aggregates of AOB were larger in the CAS compared to the MBR indicating a possible influence of the high shear forces induced by the coarse bubble aeration within the MBR. It is concluded that the membrane separation itself does affect neither the nitrifying community composition nor the nitrification performance.

Plants treating ammonium-rich wastewater – e.g. separate treatment of digester liquids – enrich nitrifiers, which could be used for bioaugmentation. However, such reactors harbor a different community composition of nitrifying bacteria that is poorly adapted to the conditions

prevailing in WWTP. It was shown that the enriched nitrifiers were not able to adapt to those conditions and rapidly were washed out after addition to the CAS and MBR pilot plants. Hence, the enriched community composition is crucial for success and failure of bioaugmentation.

Kinetics

The half-saturation constants for substrate were low in both MBR and CAS and did not differ significantly between nitrification and denitrification. On the other hand, the half-saturation constants for oxygen of the CAS were significantly higher in comparison to the MBR for both AOB and NOB. The differences were attributed to mass transfer effects within the large flocs prevailing in the conventional system. By contrast, the sludge from the MBR consisted of very small flocs where diffusion resistance can be neglected.

Decay at anoxic conditions is negligible for both AOB and NOB. No significant differences between membrane and conventional activated sludge were detected. Likewise, aerobic decay of AOB and NOB did not diverge. However, the measured loss of autotrophic activity was only partly explained by the endogenous respiration concept as incorporated e.g. in the activated sludge model no. 3 (ASM3). Unlike NOB, AOB showed a pronounced decay of their enzymes.

Conclusions

Existing models for activated sludge processes can also be applied to MBRs. However, kinetic parameters for oxygen affinity of nitrifying bacteria have to be adapted. As a consequence, MBRs can be operated at low oxygen concentrations ($1 \text{ gO}_2 \text{ m}^{-3}$) in order to save aeration energy and to enhance pre-denitrification.

The extension of activated sludge models with enzyme kinetics of AOB and the consideration of mass transfer effects within the flocs may improve our understanding of nitrogen dynamics in wastewater treatment.

Zusammenfassung

Einleitung

Während der letzten Jahrzehnte entwickelten sich kommunale Abwasserreinigungsanlagen (ARA) von der ausschliesslichen Elimination organischer Stoffe weiter zu Anlagen mit Nährstoffelimination. Damit konnte einerseits die Eutrophierung der Küstengewässer reduziert werden und andererseits nachteiligen Auswirkungen von Ammoniak vorgebeugt werden. In der Folge erhöhten sich die erforderlichen Beckenvolumina der biologischen Stufe um den Faktor 5-10. Zudem verlangen strenge Einleitbedingungen für empfindliche Oberflächengewässer eine Filtrationsstufe nach der biologischen Reinigung. Umgekehrt wird das Platzangebot in städtischen Gebieten zunehmend knapper. Gleichzeitig steigt der Druck für eine Wiederverwendung des gereinigten Abwassers in Regionen mit Wasserknappheit.

Membranbioreaktoren (MBR) zur Behandlung kommunalen Abwassers kommen seit einigen Jahren zunehmende Bedeutung zu. MBR benötigen im Vergleich zu konventionellen Belebtschlammanlagen deutlich weniger Platz, da die Nachklärung entfällt und die Becken aufgrund der höheren Belebtschlammkonzentrationen kleiner gebaut werden können. Weiter garantieren MBR einen keimfreien Ablauf. Hauptsächlich höhere Kosten und ein höherer Energieverbrauch wirken sich – neben der Unsicherheit einer neuen Technologie für den Investor – immer noch nachteilig für das neue Verfahren aus. Trotzdem ist anzunehmen, dass fallende Membranpreise aufgrund zunehmenden Wettbewerbs und sinkender Energieverbrauch durch optimierten Betrieb dem Membranverfahren zu mehr Attraktivität in den kommenden Jahren verhelfen werden. Verschärfte Einleitbedingungen und der Umgang mit Mikroverunreinigungen dürften zu einer weiteren Verbreitung von MBR führen. Membran-Kleinanlagen zur Reinigung und anschliessender Wiederverwendung des Abwassers von einzelnen Mehrfamilienhäusern sind bereits kommerziell erhältlich.

In den letzten Jahren gingen erste MBR zur kommunalen Abwasserreinigung in Betrieb. Bei der Bemessung stützte man sich auf die breite Erfahrung von konventionellen Anlagen. Allerdings fehlen langfristige praktische Erfahrungen mit MBR. Dies führt zu gewisser Vorsicht und Zurückhaltung bei den planenden Ingenieuren und Betreibern von ARA.

Ziel der Arbeit

Das Ziel dieser Arbeit war die Untersuchung des Einflusses des Membranverfahrens auf den Belebtschlamm. Dies beinhaltete das Studium der Populationsdynamik und die Erarbeitung von kinetischer Information. Die gewonnenen Erkenntnisse sollten zu einem besseren Verständnis führen, wie das breite Wissen und die langjährige Erfahrung von konventionellen Anlagen auf MBR übertragen werden können. Die Untersuchung beschränkte sich dabei auf die Nitrifikation und die beteiligten Mikroorganismen aufgrund derer zentraler Rolle in der kommunalen Abwasserreinigung.

Vorgehen

Eine konventionelle und zwei MBR Pilotanlagen wurden im Parallelbetrieb während zwei Jahren betrieben. Kommunales Abwasser mit einem typischen Tagesgang diente als Zulauf. Die konventionelle und die Membrananlagen behandelten das Abwasser von 60 ($18 \text{ m}^3 \text{ d}^{-1}$) respektive 2 ($0.56 \text{ m}^3 \text{ d}^{-1}$) Einwohnergleichwerten. Die MBR Pilotanlagen waren ausgerüstet mit je vier Hohlfasermodulen von Zenon (ZW-10). Jedes Modul hat eine spezifische Oberfläche von 0.93 m^2 und eine charakteristische Porenweite von $0.1 \mu\text{m}$. Die Anlagen wurden mit vorgeschalteter Denitrifikationszone und bei einem Schlammalter von 20 Tagen betrieben. Die Inbetriebnahme der Anlagen fand ohne externe Animpfung statt.

Die Quantifizierung der Nitrifikantenpopulation erfolgte mit Hilfe von FISH (Fluorescent *In Situ* Hybridization). Verschiedene Batchexperimente wurden durchgeführt, um die Kinetik der Nitrifikanten zu bestimmen.

Resultate

Quantifizierung nitrifizierender Bakterien

Die Quantifizierung von Bakterien mittels FISH, confocal laser scanning microscope (CLSM) und digitaler Bildauswertung ist zeitintensiv und setzt ein teures Mikroskop voraus. Deshalb wurde eine Methode entwickelt, um die Nitrifikanten im Belebtschlamm schnell und ohne CLSM zuverlässig zu quantifizieren. Die Quantifizierung des Biovolumens erfolgt durch manuelles Zählen und Bestimmen der Grösse der Nitrifikanten-Cluster unter dem Epifluoreszenz-Mikroskop. Der Zeitaufwand pro Probe beträgt im Vergleich zum oben beschriebenen Vorgehen (ca. eine Stunde) lediglich 5-15 Minuten. Die Populationsdynamik der Nitrifikanten in Belebtschlamm konnte mit der entwickelten Methode erfolgreich verfolgt werden.

Populationsdynamik

Die Bestimmung der Zusammensetzung der Nitrifikantenpopulationen mittels FISH erbrachte keine nennenswerten Unterschiede bezüglich den Ammonium- (AOB) und Nitrit-oxidierenden Bakterien (NOB) zwischen der konventionellen Anlage und den MBR. Dementsprechend waren auch die maximalen Nitrifikationsraten in beiden Systemen gleich. Untersuchungen mit dem CLSM zeigten, dass die Nitrifikanten-Cluster grösstenteils im Innern der Flocken angesiedelt sind, da sie von den schnell wachsenden, heterotrophen Bakterien überwachsen werden. Die Cluster der NOB waren allgemein kleiner als jene der AOB. Ferner waren die AOB-Cluster der konventionellen Anlage kleiner als jene der MBR. Aus den Resultaten kann geschlossen werden, dass die Membranseparierung weder die Zusammensetzung der Nitrifikantenpopulationen noch die Nitrifikationsleistung beeinflusst.

Anlagen zur Behandlung ammoniumreichen Abwassers – z. B. separate Behandlung von Faulwasser – reichern Nitrifikanten an, die grundsätzlich für die Unterstützung der

Nitrifikationsleistung in ARA eingesetzt werden könnten (auch Bioanreicherung genannt). Allerdings weisen solche Reaktoren eine deutlich andere Zusammensetzung der Nitrifikantenpopulationen auf, die schlecht an die Umweltbedingungen in ARA angepasst sind. Es wurde gezeigt, dass sich die so angereicherten Nitrifikanten nicht an die Umweltbedingungen in ARA anpassen konnten und folglich nach der Zugabe in die konventionelle und MBR Pilotanlagen sofort ausgewaschen wurden. Die Zusammensetzung der angereicherten Nitrifikantenpopulationen ist daher entscheidend für den Erfolg der Bioanreicherung.

Kinetik

In beiden Systemen wurden tiefe Monodkonstanten für Ammonium und Nitrit gefunden. Andererseits waren die Monodkonstanten für Sauerstoff im konventionellen System deutlich höher für AOB und NOB. Die Unterschiede sind zurückzuführen auf Massentransportlimitierung in den grossen Belebtschlammflocken im konventionellen System. Umgekehrt besteht der Belebtschlamm in den MBR aus kleinen Flocken, worin der Diffusionswiderstand keine Rolle spielt.

Zerfall unter anoxischen Bedingungen ist im Gegensatz zu aeroben Bedingungen vernachlässigbar für AOB und NOB. Keine signifikanten Unterschiede zwischen dem aeroben Zerfall von AOB und NOB wurden gefunden. Allerdings konnte die gemessene Abnahme der Aktivität nur teilweise mit dem Konzept der endogenen Atmung (inkorporiert z.B. im Activated Sludge Model No. 3) erklärt werden. Im Unterschied zu den NOB bauen AOB ein Teil ihrer Enzyme bei knappem Substratangebot ab.

Schlussfolgerungen

Die verfügbaren Belebtschlammmodelle können auch für MBR verwendet werden. Allerdings müssen die kinetischen Parameter für die Sauerstoffaffinität der Nitrifikanten angepasst werden. Dadurch können MBR bei tiefen Sauerstoffkonzentrationen ($1 \text{ g}_{\text{O}_2} \text{ m}^{-3}$) betrieben werden, um einerseits Belüftungsenergie zu sparen und andererseits die vorgeschaltete Denitrifikation zu steigern.

Die Erweiterung der Belebtschlammmodelle mit Enzymkinetik der AOB zusammen mit der Berücksichtigung von Massentransportlimitierung in den Flocken kann dazu beitragen, unser Verständnis für die Stickstoffdynamik in der Abwasserreinigung zu verbessern.

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Thanks are also due to the laboratory staff led by Jack Eugster. Their accurate and reliable work was an important prerequisite for the success of the experiments.

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Abbreviations

Nitrogen compounds

NH_3 ; NH_4^+ ; $\text{NH}_4\text{-N}$	Ammonia; Ammonium; Ammonium-nitrogen
NH_2OH	Hydroxylamine
NO_2^- ; $\text{NO}_2\text{-N}$	Nitrite; Nitrite-nitrogen
NO_3^- ; $\text{NO}_3\text{-N}$	Nitrate; Nitrate-nitrogen
N_2	Nitrogen gas, elemental nitrogen
Kj-N	Kjeldahl-nitrogen (sum of ammonium and organic nitrogen)

Bacteria

AOB	Ammonia-oxidizing bacteria
AMO	Ammonia monooxygenase
HAO	Hydroxylamine oxidoreductase
N.	Nitrosomonas
NOB	Nitrite-oxidizing bacteria
$\text{NO}_2\text{-OR}$	Nitrite oxidoreductase
HET; H	Heterotrophic bacteria

Others

ASM3	Activated sludge model no. 3
ATU	Allylthiourea
ATP	Adenosine triphosphate
CAS	Conventional activated sludge plant
CLSM	Confocal laser scanning microscopy
COD	Chemical oxygen demand
CV	Coefficient of variation
DOC	Dissolved organic carbon

FISH	Fluorescent <i>in situ</i> hybridization
H ₂ O ₂	Hydrogen peroxide
HCl	Hydrogen chloride
HRT	Hydraulic retention time
MBR	Membrane bioreactor
NaHCO ₃	Sodium bicarbonate, sodium hydrogen carbonate
NaOH	Sodium hydroxide
ORP	Oxidation-reduction potential
OUR	Oxygen utilization (uptake) rate
PO ₄ ²⁻ ; PO ₄ -P	Phosphate; Phosphate-phosphorus
rRNA	Ribosomal ribonucleic acid
SBR	Sequencing batch reactor
SF	Safety factor
SRT; SA	Sludge retention time; Sludge age
TSS	Total suspended solids
WWTP	Wastewater treatment plant

Kinetic parameters

b	Endogenous respiration rate
μ _{max}	Maximum growth rate
D	Diffusion coefficient
K	Half-saturation constant of Monod kinetics
θ	Temperature coefficient
r	Rate

Introduction

Introduction

Application of membranes in wastewater treatment

While membrane technology is state of the art in e.g. drinking water treatment since decades, it is applied in municipal wastewater treatment only since recent years. Membrane ultrafiltration to replace sedimentation in the activated sludge process was first described in 1969 (Stephenson, 2000). Yamamoto *et al.* (1989) reported first experiences with a hollow-fibre membrane submerged into an activated sludge reactor. A low permeate flux had to be kept in order to prevent fouling of the membrane. Operation of this unit was very similar to today's membrane bioreactors (MBRs). In the mean time, a lot of research was done particularly on the fouling issue (e.g. Fane *et al.*, 2000; Field *et al.*, 1995; Judd, 2004; Nagaoka *et al.*, 1998; Roorda and van der Graaf, 2000; Stephenson, 2000; Wintgens *et al.*, 2003). Furthermore, first full-scale plants were put into operation (Figure 1). The further technical developments and significant cost reductions have sharply increased the interest in MBR technology for municipal wastewater treatment. Compared with the traditional tubular membranes applied in the past, hollow-fibre and plate membranes require much less energy. On the other hand, membrane prices have sharply decreased in past years (Figure 2). A further reduction by 50% within the next five years is assumed (Engelhardt and Rothe, 2001). Today, mostly synthetic membranes with pore sizes between 0.04 μm and 0.4 μm are commercially used.

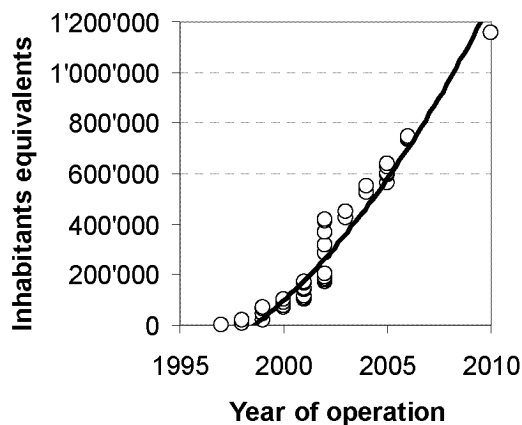


Figure 1 Full-scale MBRs worldwide.

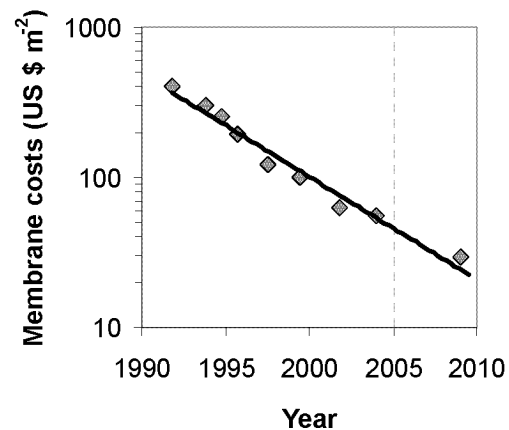


Figure 2 Development of membrane costs.

Membrane bioreactor vs. conventional system

MBR completely retain bacteria and the vast majority of viruses due to the flocculation processes and building up of a cake layer on the membrane. Therefore, a correct comparison of the MBR with the conventional activated sludge process (CAS) in terms of effluent quality must include an additional filtration and disinfection step after sedimentation (Figure 3).

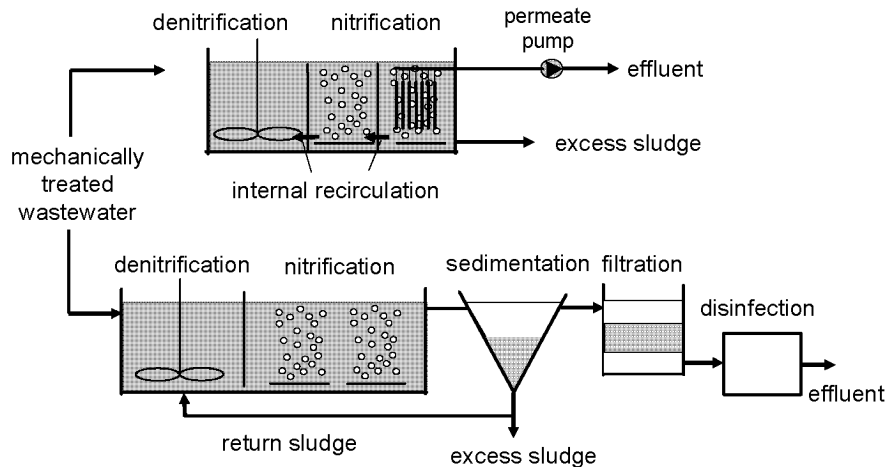


Figure 3 Comparison of a membrane bioreactor (MBR) with the conventional activated sludge process (CAS) providing similar effluent quality.

As it can be seen, MBR are particularly suited if stringent regulations (e.g. germfree effluent) have to be met and limited space is available. Other fields of application are decentralized wastewater treatment plants (WWTP) with highly dynamic load (e.g. small settlements, restaurants, apartment houses), which can be steadily operated requiring minimal monitoring effort.

Apart from the omission of the secondary settling tank, filtration and disinfection step, MBR can be operated at higher sludge concentrations due to the complete retention of solids by the membrane leading to a smaller biological volume. Alternatively, higher sludge ages can be reached at a constant volume, which may favor the degradation of slowly degradable micropollutants. However, the operation of MBR at very high sludge concentrations ($> 15 \text{ kg}_{\text{TSS}} \text{ m}^{-3}$) is not economically advantageous, because aeration efficiency and membrane performance is dramatically reduced (summarized by Wedi, 2004). Further advantages of MBR are their tolerance to shock loads because they form a highly germ-selective barrier and their operation independently of the sludge volume index. The originally expected decreased excess sludge production was not verified (Günder, 1999; Van der Roest *et al.*, 2002; Wagner and Rosenwinkel, 2000). Finally, MBR technology is suited for a flexible and phased extension of existing WWTP and is interesting in terms of an architectonic point of view.

On the other hand, mostly higher costs and little experience compared to conventional WWTP still hamper the spread of MBR. The omission of the secondary settling tank itself does not compensate by far for the costs of a MBR. However, taking into account savings for filtration and disinfection units and possible simplifications of sludge stabilization, investment costs are similar for both systems or even lower for large plants (Engelhardt and Rothe, 2001). Nonetheless, operation costs of MBR are still higher in comparison to CAS. They are dominated by the amortization of the membranes, about whose lifetime in municipal wastewater treatment can only be speculated due to lack of full-scale experience. Hitherto, lifetimes of 5 to 10 years were assumed (Wedi, 2004). A specific energy consumption of 1 kWh m^{-3} wastewater treated

was reported (Stein, 2003; Wedi *et al.*, 2004) compared to about 0.3-0.5 kWh m⁻³ for CAS. Main energy consumers of MBRs are the aeration systems, which represent about 60% of the total energy demand. Thereof accounts about 70% for the cross-flow aeration of the membranes and about 30% for the fine bubble aeration.

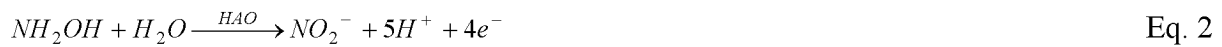
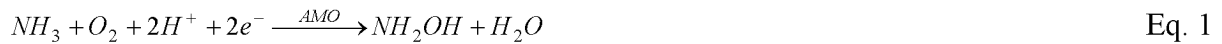
In conclusion, MBR technology still possesses optimization potential with regard to material and process engineering aspects. Increasing competence and sales volumes probably will lead to lower membrane costs. Furthermore, the coming EU directive concerning the quality of bathing waters requires the monitoring of microbiological parameters and targets a significant reduction of the germs being discharged.

Nitrification – important process within wastewater treatment

Without nitrification, municipal WWTP would discharge large amounts of ammonium (NH₄⁺) into the receiving waters. On the one hand, ammonium causes high oxygen consumption in the surface waters and on the other hand, ammonium dissociates to ammonia (NH₃) at elevated pH associated with algae growth, which is toxic to fish populations. Nitrification consists of two sequential steps: oxidation of ammonia to nitrite (nitritation) and subsequent oxidation of nitrite to nitrate (nitrataion). In each reaction, distinct groups of aerobic chemolithoautotrophic bacteria are involved. Hitherto, no autotrophic organisms are known which are able to directly oxidize ammonia to nitrate.

Nitritation

The overall reaction of ammonia to nitrite is again a two-stage process, but is catalyzed by the same organism. Ammonia rather than ammonium is used as the substrate (Suzuki *et al.*, 1974) with hydroxylamine (NH₂OH) as an intermediate (Eq. 1 and Eq. 2). The enzymes involved are ammonia monooxygenase (AMO) for ammonia oxidation and hydroxylamine oxidoreductase (HAO) to produce nitrite.



The electrons released in the hydroxylamine oxidation are required for ammonia oxidation, the respiratory chain (Eq. 3) and for CO₂ assimilation (Wood, 1986).



Combining equations 1-3 yields the overall formula for the oxidation of ammonia to nitrite (nitritation):



This reaction is catalyzed by gram-negative mostly obligate chemolithoautotrophic organisms. Although *Nitrosomonas europaea* is the model ammonia-oxidizer according to text-

books and previous studies, other ammonia-oxidizing bacteria (AOB) seem to be more important in WWTP (reviewed by Wagner *et al.*, 2002). Fluorescent *In Situ* Hybridization (FISH) analysis of various WWTP revealed that members related to *Nitrosococcus mobilis*, members of the *Nitrosomonas marina* lineage and *Nitrosomonas europaea/eutropha* were most frequently present (Purkhold *et al.*, 2000). Today it is generally accepted that nitrosomonads (including *Nitrosococcus mobilis*) and not *nitrospiras* (encompassing the genera *Nitrospira*, *Nitrosolobus* and *Nitrosovibrio*) are responsible for ammonia oxidation in WWTP. In conclusion, WWTP harbor a diversity of AOB of the *Betaproteobacteria*, which was enormously underestimated previously. Interestingly, quantitative FISH results indicate that some nitrifying WWTP are dominated by a single ammonia-oxidizer species (Juretschko *et al.*, 1998), whereas other plants harbor at least five different co-existing AOB populations, which are present in significant numbers. (Daims *et al.*, 2001b).

Nitrataion

Nitrite is further oxidized to nitrate by the enzyme nitrite oxidoreductase (NO₂-OR) and the oxygen atom in the nitrate molecule is derived from water according to Eq. 5. The two electrons released are used in the electron transport chain (Eq. 3) and partly also for CO₂ assimilation.



Traditionally, *Nitrobacter* belonging to the *Alphaproteobacteria* was considered as the most important nitrite-oxidizer in WWTP (Henze *et al.*, 2002). However, no *Nitrobacter* could be detected in samples from nine different WWTP (Wagner *et al.*, 1996), whereas the occurrence of novel, yet uncultured *Nitrospira*-like nitrite-oxidizing bacteria (NOB) in nitrifying WWTP could be demonstrated (Burrell *et al.*, 1998; Daims *et al.*, 2001a; Daims *et al.*, 2000; Gieseke *et al.*, 2001; Okabe *et al.*, 1999). Today, four different phylogenetic lineages within the genus *Nitrospira* have been recognized (Daims *et al.*, 2001a). It has been postulated that the predominance of *Nitrospira*-like bacteria over *Nitrobacter* in most WWTP is due to their higher affinity to nitrite and oxygen (reviewed by Wagner *et al.*, 2002).

An overview of the distribution and ecology of nitrifying bacteria is given in Koops and Pommerening-Roser (2001).

The well-known overall nitrification process summarizes equations 3-5 and yields Eq. 6:



Goals and questions

The underlying goal of this project was to obtain a more fundamental understanding about the effect of the membrane separation on the activated sludge properties. The new insights should help to understand how the broad knowledge and experience from CAS systems can be transferred to MBR systems. The following questions should be addressed:

- How can nitrifying bacteria reasonably be quantified in activated sludge?
- How does the membrane separation influence the populations of nitrifying bacteria?
- How does the membrane separation influence the floc structure? What are the consequences for the kinetics of nitrification?
- Does the decay of nitrite-oxidizing bacteria differ from that of ammonia-oxidizing bacteria? What is the effect of different oxidation-reduction potentials on decay?
- How is the competition among different groups of nitrifying bacteria, e.g. bioaugmentation? Are MBR systems advantageous in this regard?
- Can existing activated sludge models reliably be applied to membrane bioreactors? Which adaptations or extensions are necessary?

Outline of this thesis

The study of population dynamics presumes an adequate method to quantify the organisms of interest. **Chapter 1** presents a procedure how nitrifying bacteria in activated sludge can be reliably quantified with a reasonable effort. The method is based on Fluorescence *In Situ* Hybridization (FISH) and epifluorescence microscopy.

The influence of the membrane separation on the populations of nitrifying bacteria is described in **chapter 2** using the method developed in chapter 1. The startup behavior and the performance of the subsequent eight months of a MBR are compared with a CAS running in parallel, tracking the evolution of ammonia- and nitrite-oxidizing bacteria. Special emphasis was placed on the strict separation of the influence of the membrane from other factors e.g. SRT or influent composition.

Bioaugmentation is often suggested as a concept to improve the nitrification performance without the need of enlarging the aeration tanks. In **chapter 3** the potential of bioaugmentation using enriched nitrifying sludge from the separate treatment of digester liquids is evaluated involving the investigation of the co-existence and competition among different nitrifying groups.

Chapter 4 demonstrates the impact of the membrane separation on the sludge structure. Based on floc size distribution measurements and batch experiments, the effects of mass transfer limitation within the activated sludge flocs were studied and their consequences for modeling of nitrification are highlighted.

Decay processes play an important role with increasing sludge ages characteristically for MBRs. Therefore, a closer look at the decay kinetics of nitrifying bacteria is presented in **chapter 5**. Differences between MBRs and CAS, ammonia- and nitrite-oxidizing bacteria as well as the effect of the oxidation-reduction potential were investigated.

Major results of this thesis are briefly summarized in the **conclusions**.

CHAPTER 1

A Rapid Method to Quantify Nitrifiers in Activated Sludge

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A Rapid Method to Quantify Nitrifiers in Activated Sludge

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Abstract

Quantification of bacteria using Fluorescence In Situ Hybridization (FISH), confocal laser scanning microscopy (CLSM) and image analysis is very time consuming and requires the availability of an expensive microscope. Therefore, a rapid method to quantify nitrifying bacteria in activated sludge using FISH and epifluorescence microscopy was developed. The quantification of the biovolume is based on manual counting of the aggregates formed by nitrifying bacteria and determination of their size. The overall uncertainty of the method was evaluated as a function of the number of analyzed microscopic fields. It was found that 10-15 microscopic fields for ammonia-oxidizing bacteria and 6-8 microscopic fields for nitrite-oxidizing bacteria per sample were optimal regarding effort and accuracy. Accordingly, the time needed for one sample was only 5 to 15 minutes, compared to about one hour for the quantification with CLSM and image analysis. As a consequence, this method also allows for the measurement of extended time series with a reasonable effort. The comparison of the determined biovolume and the measured activity showed an explicit correlation.

Keywords: Activated sludge; FISH; Nitrifying bacteria; Quantification; Epifluorescence microscopy

1. Introduction

Increasing demands on wastewater treatment in the last decades led to larger and more sophisticated treatment plants. The extension of the biological treatment steps from only COD removal to plants with nitrification, denitrification and biological phosphorus removal implied higher sludge retention times and more complex ecosystems. As a consequence, operating problems due to scumming and bulking sludge are increasing. Stable biological phosphorus removal can often not be maintained. Furthermore, nitrification failure and the potential of bioaugmentation to enhance nitrification performance are still to be explored.

Until now, these issues could be only inadequately addressed with traditional engineering methods, e.g. respirometry and light microscopy. New technical approaches in molecular microbiology such as Fluorescence *In Situ* Hybridization (FISH) permit a new level of insight into the population structure and function of complex ecosystems like activated sludge (Juretschko et al., 2002; Wagner et al., 2002; Wagner et al., 1995). This molecular biological tool opens up new vistas for the solution of the above mentioned issues. However, a couple of questions remain unanswered when applying the FISH technique.

1. For most applications bacteria have to be quantified. Previously, quantification was mainly done with confocal laser scanning microscopy (CLSM) followed by image analysis (Bouchez

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et al., 2001; Daims *et al.*, 2001c; Kuehn *et al.*, 1998). This method focuses on the biovolume and uses a threshold signal intensity to discriminate between target and non-target cells. The biovolume of the specific bacteria of interest is then referred to the biovolume of all bacteria, detected by a general probe (e.g. EUB338; Amann *et al.*, 1990). However, several constraints have to be considered. Firstly, the availability of an expensive microscope is needed. The image quality of thick samples like activated sludge flocs by conventional epifluorescence microscopy does not allow a subsequent image analysis unless the samples are physically sectioned (Wagner *et al.*, 1998). Secondly, the technique is time consuming, which makes the analysis of long time series tedious and hardly feasible. Thirdly, despite the semi-automatic procedure using image analysis, the threshold setting is still a subjective task which has to be done either directly at the microscope or when analyzing the images. Problems with inhomogeneous signal intensities may hamper the threshold setting (Daims *et al.*, 2001c). Hence, cells with low activity and therefore low signal intensity are excluded. Moreover, debris particles showing high signal intensities are not omitted and would have to be deleted manually in the image analysis step.

2. Other methods assign the abundance of filamentous bacteria to categories based on reference images (Eikelboom and van Buijsen, 1983; Jenkins *et al.*, 1993). This technique was recently enhanced using FISH and epifluorescence microscopy (Hug *et al.*, 2005). However, only large differences can be detected. In contrast to filaments, nitrifiers mainly appear as dense aggregates within activated sludge flocs (Wagner *et al.*, 1995). The size of these aggregates is crucial regarding the abundance. Therefore, the quantification of nitrifiers based on reference images is not possible. For the same reason, manual counting of single cells or quantification by flow cytometry are not feasible.

3. Uncertainties of the quantification with regard to the sampling, the method and the operator must be determined in order to identify the main sources of error. Since a higher accuracy of the results involves a higher time demand, the uncertainty analysis allows the optimization of work to meet the individual criteria.

4. It has to be clarified whether one should quantify cell numbers, biovolume or activity (measured as intensity of the probe signal). While traditionally cell numbers were determined, new quantification methods measure the abundance as biovolume. The consideration of the probe signal intensity, which is proportional to the rRNA content of the cells and therefore their activity, has still to be explored. However, nitrifiers seem to keep their rRNA content also during periods with low activity (Wagner *et al.*, 1995). Consequently, the signal intensity does not stringently reflect the actual metabolism activity.

5. It is not clear, how to link these numbers with measured parameters such as COD, TSS or activity (measured e.g. as ammonia oxidation rates). Hall *et al.* (2003) compared the *Nitrospira* fraction quantified by FISH and CLSM to measured nitrate production rates without finding a clear correlation between the biovolume and the rate. Thus, the link to the above mentioned parameters has still to be evaluated.

The goal of this study was the development of a rapid method to quantify nitrifying bacteria in activated sludge without the use of an expensive CLSM. Although many researchers might have some experience with manual quantification using epifluorescence microscopy, no reproducible methods have been properly developed so far. Especially in engineering systems like activated sludge processes with low enrichment of nitrifying bacteria, a reasonably fast and reliable quantification method is needed to follow population dynamics, e.g. during bio-augmentation studies. Furthermore, the quantitative microbiological information has to be linked to measured activity in order to evaluate its potential for modeling purposes.

2. Materials and Methods

Activated sludge samples used for the uncertainty analysis were taken from a membrane bioreactor (MBR) and a conventional activated sludge (CAS) pilot plant treating domestic wastewater (Manser *et al.*, 2004). Activity measurements were done using sludge from the MBR and a sequencing batch pilot reactor (SBR, volume 2 m³) inoculated with sludge from the SBR for the separate treatment of digester liquid at the wastewater treatment plant (WWTP) of Bülach (Switzerland).

2.1 Measurement of the activity (Batch experiments)

A) Sludge from the MBR. A batch reactor (Volume 1 liter) was inoculated with diluted activated sludge and continuously aerated. Ammonium and oxygen concentrations were always above 10 g_N m⁻³ and 4 g_{O₂} m⁻³, respectively. Nitritation rates (conversion of ammonia to nitrite) were regularly measured by titration with NaOH (Massone *et al.*, 1998). Samples were taken simultaneously for the analysis with FISH. The experiment was performed at pH 7.5 and 20°C.

B) Sludge from the SBR. The nitritation rate was measured once a day by means of respirometry (Drtil *et al.*, 1993) in a batch reactor (Volume 7.5 liter). Ammonium and oxygen concentrations were always above 100 g_N m⁻³ and 4 g_{O₂} m⁻³, respectively. Samples were taken simultaneously for the analysis with FISH. All measurements were performed at pH 7.5 and 19°C.

2.2 Fluorescence In Situ Hybridization (FISH)

Samples were fixed in 4% paraformaldehyde as described by Amann *et al.* (1990). Ultrasonication (35 kHz, five minutes) was applied to the fixed samples from the CAS and the SBR prior to hybridization in order to break up large flocs. *In situ* hybridizations of cells were performed with fluorescently labeled (Cy3), rRNA-targeted oligonucleotide probes according to Manz *et al.* (1992) (Table 1). Oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland) and Thermo Hybaid (Interactiva Division, Ulm, Germany).

Table 1 Oligonucleotide probes and hybridization conditions applied in this study

Probe	Target organisms	Reference
Ntspa662 ^a	Genus <i>Nitrospira</i>	Daims et al., 2001a
NEU ^a	Most halophilic and halotolerant ammonia oxidizers in the beta-subclass of <i>Proteobacteria</i>	Wagner et al., 1995
NmII	Many members of the <i>Nitrosomonas communis</i> lineage	PommereningRoser et al., 1996
Nmo218	Many members of the <i>Nitrosomonas oligotropha</i> lineage	Gieseke et al., 2001
Nso1225	All known ammonia oxidizers in the beta-subclass except <i>N. mobilis</i>	Mobarry et al., 1996

^a used with unlabeled competitor as indicated in the reference

2.2 Microscopy

All samples hybridized with oligonucleotide probes were embedded in Citifluor (Citifluor, Canterbury, United Kingdom) prior to microscopic observation. Epifluorescence microscopy was done using an Olympus BX50 microscope, equipped with a filter HQ-CY3 (Analysentechnik AG, Tübingen, Germany). Microscopic fields (diameter: 200 µm) were analyzed with 1000x magnification. For quantification an ocular with an implemented grid of 10 µm equidistance was used (Figure 1).

2.3 Quantification

Microscopic fields were randomly chosen by the operator. Since the thickness of the sample on the slide varied from field to field, only one focus plane instead of the complete depth of each microscopic field was analyzed. Positively labeled aggregates were manually counted and the average diameter of every aggregate was determined with the aid of the grid (Figure 1). Accordingly, dirt particles could be easily excluded and a large range of probe signal intensities could be taken into account.

The biovolume instead of the bioarea was used to allow linking the abundance to activity measurements and to estimate single cell rates. It was calculated assuming spherical shape of the aggregates, which was true for the vast majority of aggregates. The compression on the slide was found to be negligible for aggregates with diameters below 20 µm, using the ruler on the fine focus wheel to estimate their vertical dimension. Single cells were approximately counted and assigned a diameter of 1 µm and 0.7 µm for ammonia- and nitrite oxidizing bacteria, respectively. Digital images of single cells were taken in order to estimate their mean diameter. However, single cells only contributed to a negligible extent to the abundance.

As reference system, the area occupied by the activated sludge floc was chosen. In addition to the counting of the aggregates, the coverage *c* of every microscopic field by the flocs – visible as blurry auto-fluorescence signal of the sludge – was estimated (Figure 1). The variation of the biomass density within the flocs was considered as part of the overall uncertainty.

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The abundance (as biovolume per floc cross-section) was calculated using the following formula:

$$bv_{floc} = \frac{bv_{field}}{c \cdot A_{field}} [\mu m^3 mm_{floc}^{-2}]$$

with: bv_{floc} = biovolume per floc cross-section [$\mu m^3 mm_{floc}^{-2}$]

bv_{field} = biovolume per microscopic field [$\mu m^3 field^{-1}$]

c = coverage of microscopic field with flocs [$mm_{floc}^2 mm_{field}^{-2}$]

A_{field} = area of microscopic field [$mm_{field}^2 field^{-1}$]

At first glance, the dimension of the abundance as volume per area may be confusing. This reference system was chosen, since the purpose of this method is to provide a good reproducibility of the results rather than absolute numbers based on a certain reference system. Furthermore, the correlation of the biovolume with measured activity is independent of the applied reference system.

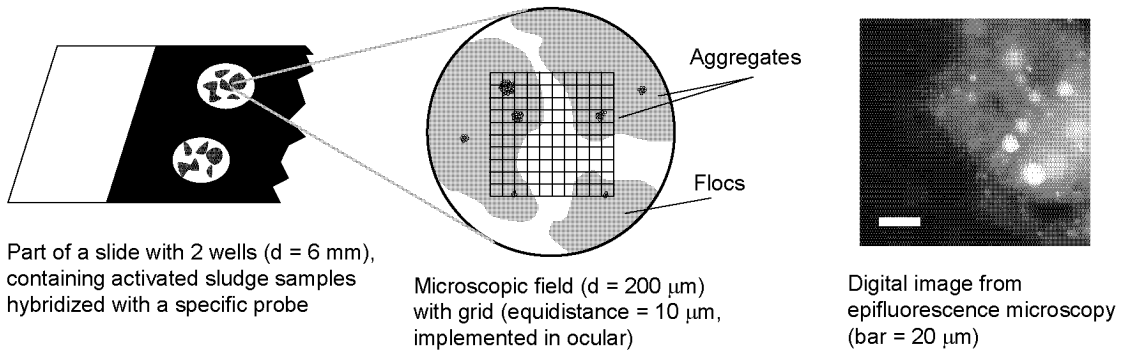


Figure 1 Principle and nomenclature of quantification procedure

2.4. Uncertainty analysis

The uncertainty analysis provides quantitative data about the accuracy and reliability of the method, which is crucial for its application. The overall uncertainty of the quantification method was determined by analyzing 45 to 55 fields for each probe (FISH), using the same activated sludge sample (MBR). However, the values gained (bv_{floc}) represent only a random sample of the complete distribution. Since the complete distribution is not known and cannot be assigned from a priori knowledge, the nonparametric bootstrap method was applied (Efron and Tibshirani, 1993). This statistical method allows estimating an empirical distribution without the assumption of normality. Basically, for an entity of N values, a bootstrap sample b is obtained by randomly sampling N times with replacement. In order to evaluate the overall uncertainty as a function of the number of analyzed fields, $B = 10'000$ bootstrap samples for every probe ($N = 45$ to 55) were generated. Subsequently, the estimated expected value $\hat{\mu}_n$, standard error \hat{se}_n and coefficient of variation CV_n were calculated using the following formulas:

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$$\hat{\mu}_n = \frac{\sum_{b=1}^{B=10'000} \hat{\mu}_{n,b}}{B}, \quad \hat{\mu}_{n,b} = \frac{\sum_{i=1}^n bv_{i,b}}{n}$$

$$\hat{se}_n = \sqrt{\frac{1}{B-1} \sum_{b=1}^{B=10'000} (\hat{\mu}_{n,b} - \hat{\mu}_n)^2}, \quad CV_n = \frac{\hat{se}_n}{\hat{\mu}_n}$$

with: $n = 1 \dots N$: number of microscopic fields
 $b = 1 \dots B$: number of bootstrap sample
 $bv_{i,b}$ = biovolume per floc cross-section of field i and bootstrap sample b
 $\hat{\mu}_{n,b}$ = estimated expected value of bootstrap sample b
 $\hat{\mu}_n$ = estimated expected value when analyzing n fields
 \hat{se}_n = estimated standard error when analyzing n fields
 CV_n = coefficient of variation when analyzing n fields

3. Results

The result of interest, namely the estimation of nitrifier biovolume in the activated sludge, is affected by several sources of uncertainty. They are caused by the measurement procedure and mainly stem from:

1. the missing representativeness of the grab sample
2. the FISH protocol (fixation, hybridization)
3. the natural spatial variability of the aggregates and their subjective estimation
4. systematic errors among different operators

The first two sources will be excluded in the further considerations, because the samples used were taken from completely mixed pilot reactors and the FISH protocol was standardized. However, the representativeness of samples from full scale plants should be examined by tracer experiments, which allow for the assessment of the hydraulics of the plant and the detection of insufficiently stirred zones. The latter two sources are assessed in sections 3.1 and 3.2.

3.1 Natural spatial variability of the aggregates and their subjective estimation

For every probe between 60 and 600 aggregates were enumerated by the same operator. The average cell diameter of ammonia-oxidizing bacteria (AOB: probes Nso1225, Nmo218, NEU and NmII) was 1-1.2 μm . While cells detected by the NEU probe were present in low numbers (~ 1 aggregate per microscopic field) and rather heterogeneously distributed, cells detected by Nmo218 made up the major fraction (~ 4 aggregates per microscopic field) of AOB. The diameter of the aggregates ranged up to 16 μm . In contrast, aggregates of nitrite-oxidizing bacteria (NOB: probe Ntspa662) were generally smaller (up to 8 μm). However, the

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cell diameter of NOB as detected by probe Ntspa662 was also smaller (0.5-0.8 μm). About 8 aggregates per microscopic field were enumerated on the average.

The observed differences of the spatial distribution and size of the aggregates influenced the frequency distribution of the measured abundances (Figure 2). All groups of AOB showed a wide distribution. The asymmetric pattern reflects the transformation from diameters to bio-volume (spherical aggregates). Moreover, some fields with abundances far higher than the average value further pronounced this effect. Despite this transformation, NOB showed a rather symmetric pattern, which can be explained by the general smaller aggregate size and their more homogeneous spatial distribution compared to the AOB.

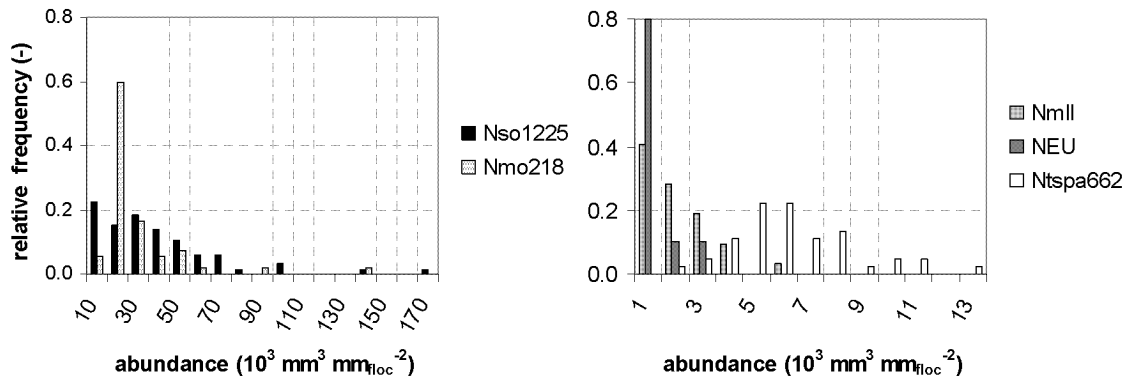


Figure 2 Histograms of the series used for the estimation of the uncertainties. 55 microscopic fields (Ntspa662: 45) were analyzed for each probe using the same activated sludge sample (MBR). AOB: Nso1225, Nmo218, NmII, NEU; NOB: Ntspa662.

The overall uncertainty of the mean abundance as a function of the number of microscopic fields was estimated using the bootstrap method (Figure 3). Depending on the required accuracy of the quantification, Figure 3 allows the determination of the number of fields that need to be analyzed. As it could be expected from the distributions (Figure 2), there are large differences between the detected groups, especially between ammonia- and nitrite-oxidizing bacteria. As an illustration, at 10 microscopic fields the estimated CV is 0.33 for the Nso1225 probe, however, this value is only 0.10 for the Ntspa662 probe, which reflects the pronounced difference between the spatial variability of AOB and NOB.

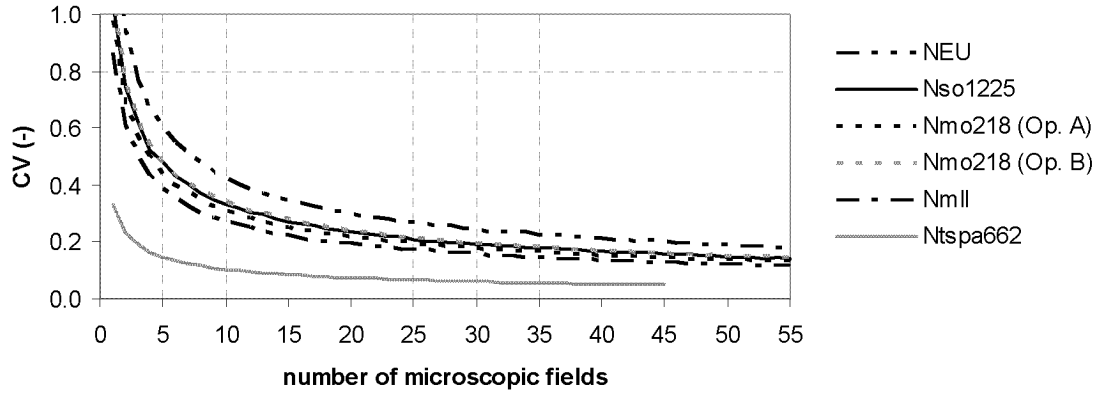


Figure 3 Estimation of the overall uncertainty of the mean abundance by the bootstrap method. CV = coefficient of variation. Probe Nmo218 was analysed twice by two operators (Op. A and B) as described in section 3.2.

3.2 Systematic errors among different operators

Two main sources of systematic errors due to the subjectivity of the operator were identified and assessed. Systematic deviations of the mean abundance stem either from the subjective estimation of the number and the size of the aggregates or from the subjective choice of microscopic fields.

Firstly, the number and size of the counted aggregates could be significantly higher or lower among different operators. Therefore, 10 microscopic fields (stained with probe Nmo218) were analyzed by five operators (Figure 4). Three of them were using this method for the first time. A Kruskal-Wallis test (nonparametric version of one-way analysis of variance) showed that no significant differences among the operators were found ($p = 0.98$). However, the CV for each quantified microscopic field varied between 0.01 and 0.57 (average: 0.22). As a consequence, the differences between the operators for each quantified microscopic field also had a high variance, which hampers the identification of systematic effects.

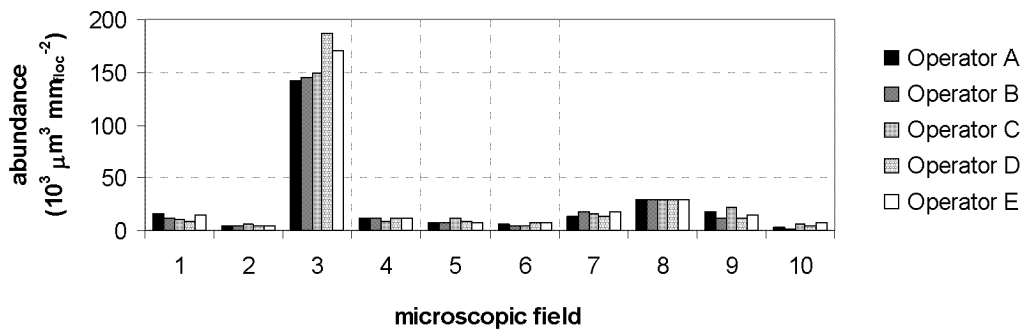


Figure 4 Results from the analysis of systematic errors due to the estimation of the number and size of the aggregates (Probe Nmo218).

Secondly, systematic errors among operators are introduced because the microscopic fields may not be randomly chosen. Therefore, 11 wells (stained with probe Nmo218), encompass-

ing 6 microscopic fields each, were analyzed twice. The analyzed fields were chosen by Operator A in the first run and by Operator B in the second run. Operator A quantified all fields in both runs in order to prevent mixing systematic effects due to the choice of the fields and due to the estimation of number and size of the aggregates. A signed rank test indicates that the effect of the choice of the microscopic fields did not lead to significant differences between the two persons ($p = 0.31$). However, the high spatial variability involved pronounced deviations for single wells (Figure 5). The main reason are large aggregates, which strongly influence the result due to the spherical shape of the aggregates. For example, while Operator A hit one large aggregate (diameter $18\ \mu\text{m}$) in well 11 (Figure 5), the fields chosen by Operator B did not contain this aggregate. The calculation of the average value over the 66 microscopic fields yielded 15.3 ± 1.8 and $13.5 \pm 1.8 \times 10^3\ \mu\text{m}^3\ \text{mm}_{\text{floc}}^{-2}$ for Operator A and B, respectively. An almost identical shape of the overall uncertainty of the mean abundance was found for the two series (Figure 3).

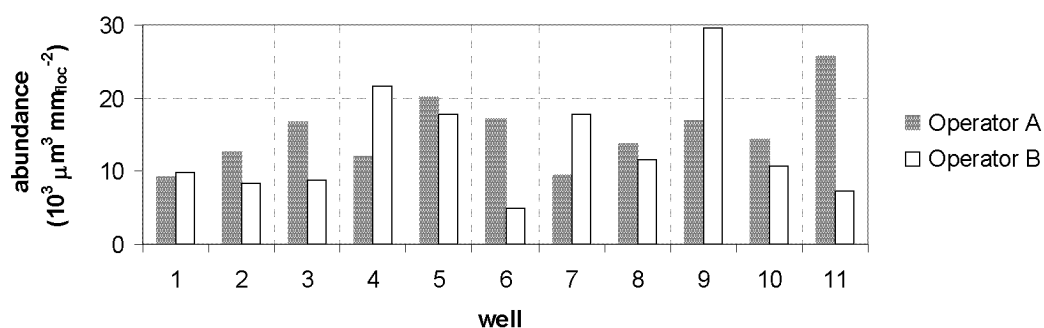


Figure 5 Results from the analysis of systematic errors due to the choice of the microscopic fields. 6 microscopic fields were analyzed per well (Probe Nmo218).

3.3 Time demand for quantification

Depending on the abundance of the aggregates, the time demand for the analysis of one microscopic field ranged from 20 seconds (probe NEU) to 90 seconds (probe Ntspa662) on the average for operators familiar with the FISH technique and epifluorescence microscopy.

3.4 Link between abundance of nitrifiers and measured activity

In activated sludge models, biomass concentrations are estimated by measuring their activity. By contrast, quantitative FISH is a direct measure of the biomass concentration. The combination of the information about biomass concentrations and their activity provides new insights into inhibition of bacteria and competition among different bacteria. Two activated sludges with different populations of AOB were examined. The sludge from the MBR pilot plant was dominated by members of the *Nitrosomonas oligotropha* lineage (probe Nmo218), whereas members of the *Nitrosomonas eutropha/europaea* lineage (probe NEU) were the main representatives of the AOB population in the sludge from the SBR. The comparison of measured nitrification rates (conversion of ammonia to nitrite) with the biovolume of AOB revealed a lin-

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ear relationship for both the MBR (Figure 6A, $r = 0.96$) and the SBR plant (Figure 6B, $r = 0.94$). A weighted linear regression yielded significant values for the slope ($p = 0.008$ and 0.02 for the MBR and SBR, respectively) but not for the intercept ($p = 0.37$ and 0.34). Under the assumptions that the biovolume of one cell is $0.5\text{--}1\ \mu\text{m}^3$ and $1\ \mu\text{l}$ of fixed sample consists of 10–30 microscopic fields (depending on the sludge concentration), specific nitrification rates per cell can be estimated. The calculation yielded $1\text{--}7 \times 10^{-3}$ and $3\text{--}18 \times 10^{-3}\ \text{pmol cell}^{-1}\ \text{h}^{-1}$ for the MBR and the SBR plant, respectively. Assuming a biomass density of $1600\ \text{kg}_{\text{SS}}\ \text{m}^{-3}$ (Mueller et al., 1966), the corresponding values are $0.3\text{--}2.0$ and $0.8\text{--}5.0\ \text{kg}_\text{N}\ \text{kg}_{\text{SS}}^{-1}\ \text{d}^{-1}$ for the MBR and the SBR plant, respectively.

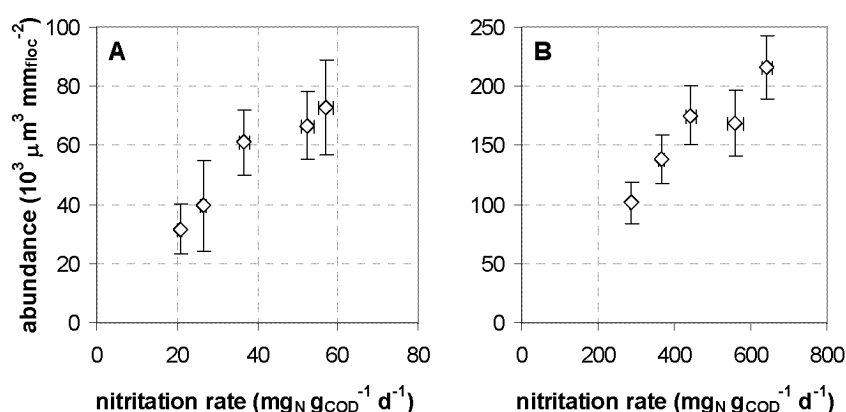


Figure 6 Relationship between measured nitrification rate and abundance of AOB. A) Sludge from the MBR pilot plant. 12 microscopic fields were analyzed for each sample (probe Nso1225). B) Sludge from the SBR. 14 microscopic fields were analyzed for each sample (sum of probes NEU, Nmo218 and NmII). Error bars indicate one standard error.

4. Discussion

Although the FISH technique is nowadays often applied to engineering systems such as wastewater treatment plants, its benefits for practical applications are limited. On the one hand, fast and reliable methods to quantify bacteria are needed to process large number of samples. On the other hand, the quantitative data must be linked to traditional parameters such as substrate consumption rates which allows its use for modeling.

This study focuses on the nitrifying population, because nitrification is an important step within the treatment of wastewater. Moreover, nitrifiers are vulnerable to perturbations and are of interest for bioaugmentation studies. Their generally low abundance in activated sludge and their appearance in densely packed aggregates facilitate their quantification without the need of an expensive CLSM with subsequent image analysis.

The new quantification method was successfully applied to reproducibly determine the abundance of nitrifiers in activated sludge. Although uncertainties due to the natural spatial variability of the aggregates and their subjective estimation were not separated in the analysis, the results revealed that the main part of the overall uncertainty arises from the spatial variability

of the aggregates within the activated sludge. The analysis did not show significant deviations among different operators, albeit the presented method implies several tasks which are prone to subjectivity. Systematic errors were found neither in the estimation of the number and the size of the aggregates nor in the choice of microscopic fields.

Due to the high spatial variability of AOB, many microscopic fields must be analyzed to obtain accurate mean values – independently of the quantification method applied. Below 10 microscopic fields every additional field leads to a large increase of the accuracy of the result, whereas every additional field above 10 microscopic fields only slightly improves its accuracy. Therefore, an optimum between effort and accuracy of the result was found to be 10 to 15 microscopic fields per probe involving CVs of the overall uncertainty from 35 to 22% depending on the probe used. This corresponds to a time demand of 5 to 15 minutes per sample and probe. Since AOB targeted by the NEU probe were only present in very low numbers in the analyzed sample, there were many fields without any stained bacteria. Consequently, the spatial variability was higher than for the other groups of AOB examined (Figure 3). The much lower spatial variability of NOB led to an optimum of 6 to 8 microscopic fields per sample involving CVs of the overall uncertainty from 13 to 11%. Due to the higher time demand per microscopic field for NOB, approximately 10 minutes are needed per sample. Quantification by CLSM and image analysis typically takes about one hour per sample.

These accuracies were shown to be sufficient to track population dynamics in activated sludge plants (Manser *et al.*, 2004). A higher accuracy involving also a higher time demand would be required when using these results for modeling purposes. However, the time demand is still low compared to activity tests in batch experiments.

The clear correlation between the abundance and the nitrification rate of AOB suggests that the biovolume is a reasonable measure for activity. The choice of the area covered by the floc as reference system gives reproducible results. However, all measurements were carried out at a state of growth supporting cell divisions. Since nitrifiers are known to keep their ribosome content also at periods with low activity (Wagner *et al.*, 1995), alternating stages of growth and starvation would possibly alter the correlation between abundance and activity. The estimated specific nitrification rates of 1.7×10^{-3} pmol cell⁻¹ h⁻¹ (MBR) and 3.18×10^{-3} pmol cell⁻¹ h⁻¹ (SBR) agree with values found in literature, ranging from 11.23×10^{-3} pmol cell⁻¹ h⁻¹ for pure cultures of *Nitrosomonas europaea* to 0.95×10^{-3} pmol cell⁻¹ h⁻¹ for *Nitrospira* in a incubated biofilm culture (various in Prosser, 1989; Schramm *et al.*, 1999). The difference of the specific nitrification rates between the two examined sludges reflects their population composition. While members of the *Nitrosomonas eutropha/europaea* lineage (prevailing in the SBR) exhibit high maximum growth rates and a low affinity to the substrate, members of the *Nitrosomonas oligotropha* lineage (prevailing in the MBR) have lower maximum growth rates combined with a high affinity to the substrate (Koops and Pommerening-Roser, 2001; Prosser, 1989).

When applying this method, a preliminary analysis of about 50 microscopic fields for every probe targeting AOB and about 30 microscopic fields for every probe targeting NOB of a

typical sample are recommended. The subsequent uncertainty analysis as described above allows defining the number of microscopic fields needed to be analyzed per sample depending on the required accuracy. The uncertainty of the result for every sample is then expressed as standard error of the mean. Mean values rather than the median must be used because multiple fields without any targeted cells can occur for bacteria with low abundance resulting in a median of zero, although there are fields containing targeted cells.

This study has only dealt with uncertainties encompassing the quantification at the microscope. However, several preliminary steps have to be performed within the whole FISH procedure. While small modifications of the fixation and hybridization steps probably have only minor influence on the result, the sampling is more prone to uncertainties due to inhomogeneities, which are caused by incomplete mixing of the activated sludge tanks. The estimation of the overall uncertainty in this method requires homogeneity of the samples. Furthermore, the method cannot be applied to activated sludge containing very dense flocs, which do not break up during the treatment with ultrasonic.

5. Conclusions

The presented method allows fast and reliable quantification of nitrifiers in activated sludge without the need of an expensive confocal laser scanning microscope (CLSM). Moreover, it is applicable for all users of FISH. The overall uncertainty of the method is dominated by the large spatial variability of the nitrifying bacteria. However, the method was shown to be robust in terms of the subjective choice of the microscopic fields as well as the subjective estimation of the size and number of the aggregates formed by nitrifying bacteria. For the examined activated sludge, 10-15 microscopic fields for AOB and 6-8 microscopic fields for NOB involving a time demand of about 5-15 minutes per sample are found to be optimal regarding effort and accuracy, compared to about one hour using CLSM and image analysis. As a consequence, this method is a helpful tool for the evaluation of bioaugmentation strategies of nitrification or inhibition of nitrifying bacteria which imply the analysis of large time series. The demonstrated correlation between the biovolume and the measured activity of the nitrifying bacteria facilitates the application of FISH for modeling. Moreover, the presented method is far quicker than activity tests in batch experiments and could therefore partly replace them. Furthermore, the measurement of the abundance can be done at a later stage. This study has made a step towards the implementation of molecular biological techniques into engineering fields like modeling wastewater treatment plants.

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CHAPTER 2

Membrane Bioreactor vs. Conventional Activated Sludge System - Population Dynamics of Nitrifiers

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Membrane Bioreactor vs. Conventional Activated Sludge System - Population Dynamics of Nitrifiers

Reto Manser, Willi Gujer and Hansruedi Siegrist

Abstract

Although membrane bioreactors have attracted increasing attention in recent years, little research has been undertaken on the influence of the membrane separation on the microbial community composition. This paper compares the startup behaviour and the performance of the subsequent eight months of a membrane bioreactor with a conventional activated sludge pilot plant. Both plants were operated in parallel at the same sludge age and treated the same domestic wastewater. The identification of the nitrifying community composition using fluorescent in situ hybridization (FISH) revealed only minor differences between the two reactors for both ammonia-oxidizing bacteria and nitrite-oxidizing bacteria. Accordingly, both systems exhibited the same maximum nitrification rates. Confocal laser scanning microscopy showed that the aggregates formed by nitrifying bacteria were located mostly in the inner part of the flocs and were overgrown by heterotrophic bacteria. It is concluded that the membrane separation itself does affect neither the nitrifying community composition nor the nitrification performance. However, impacts on kinetic parameters are emphasized.

Keywords: CLSM; Cluster; Conventional activated sludge system; FISH; Membrane bioreactor; Nitrifying bacteria; Population dynamics

1. Introduction

Membrane bioreactors (MBR) for the treatment of municipal wastewater have attracted increasing attention in recent years. Compared to the conventional activated sludge process (CAS) they have a lower footprint due to the omission of the secondary settling tank, need smaller biological tanks and provide the effluent free of solids. First full-scale plants were put into operation in past years, adapting the experiences from conventional activated sludge plants.

However, the correct application of existing models from the conventional activated sludge process for MBR systems requires additional information about the population composition and kinetics of activated sludge in MBR. The few investigations done so far on the comparison of the two systems were carried out at different sludge ages or even without any sludge withdrawn, with synthetic or special industrial feed, fully aerobic or a combination of these factors (Cicek *et al.*, 1999; Luxmy *et al.*, 2000; Witzig *et al.*, 2002). While Luxmy *et al.* (2000) found significantly different bacterial communities in the two systems, Witzig *et al.* (2002) showed that the membrane sludge population was more substrate-limited than con-

ventional activated sludge. However, these differences are probably only due to the high sludge age, since the MBR were operated without any sludge withdrawn.

In contrast to previous research, the goal of this study was to investigate the impact of the membrane separation on the community composition at genus level (nitrifying bacteria) and on the nitrification performance. Therefore a MBR and a CAS pilot plant with the same settings were operated in parallel. The sludge age was equal in both plants in order to strictly separate the influence of the membrane from the sludge age. The treatment of domestic wastewater with diurnal variation provided real conditions.

2. Material and Methods

2.1 Pilot plants

The pilot plants (Figure 1) were fed with municipal wastewater showing a typical diurnal hydraulic variation. The CAS and the MBR treated the wastewater corresponding to 60 ($18 \text{ m}^3 \text{ d}^{-1}$) and 2 ($0.56 \text{ m}^3 \text{ d}^{-1}$) population equivalents, respectively. The MBR was equipped with 4 submerged hollow fibre modules from Zenon (ZW-10) with a surface area of 0.93 m^2 each and a characteristic pore size of $0.1 \mu\text{m}$. Coarse bubble aeration was intermittently operated according to the specifications of the manufacturer. A fine screen of 2 mm retained hairs from deteriorating the membrane performance. Oxygen concentration in the aerobic tanks was controlled between 2.5 and 3 g m^{-3} . Due to the coarse bubble aeration, the oxygen concentration was temporarily higher in the MBR. Both plants were operated with pre-denitrification and the sludge age (SA) was kept at 20 days (aerobic SA was 10 days in both systems due to higher TSS in MBR). The startup of both MBR and CAS was done without inoculation.

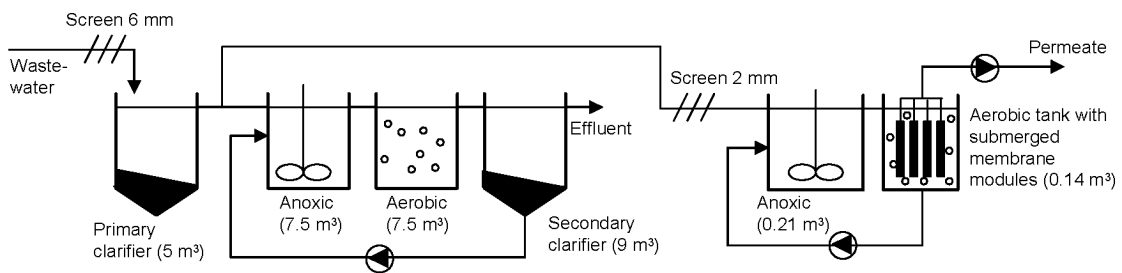


Figure 1 Setup of pilot plants (left: conventional activated sludge plant, right: membrane bioreactor)

2.2 Fluorescent *In Situ* Hybridization (FISH) and microscopy

Samples from the CAS and the MBR were fixed as described by Amann *et al.* (1990). In situ hybridizations of cells were performed with fluorescently labeled, rRNA-targeted oligonucleotide probes according to Manz *et al.* (1992). Oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland) and Thermo Hybaid (Interactiva Division, Ulm, Germany). The probes used are listed in Table 1.

Table 1 Oligonucleotide probes and hybridization conditions applied in this study

Probe	Target organisms	Reference
EUB338	Most <i>Bacteria</i>	Amann <i>et al.</i> , 1990
EUB338-II	<i>Planctomycetales</i> and other <i>Bacteria</i> not detected by EUB338	Daims <i>et al.</i> , 1999
EUB338-III	<i>Verrucomicrobiales</i> and other <i>Bacteria</i> not detected by EUB338	Daims <i>et al.</i> , 1999
Nit3 ^a	<i>Nitrobacter</i> spp.	Wagner <i>et al.</i> , 1996
Ntspa662 ^a	Genus <i>Nitrospira</i>	Daims <i>et al.</i> , 2001a
Ntspa712 ^a	Phylum <i>Nitrospira</i>	Daims <i>et al.</i> , 2001a
NEU ^a	Most halophilic and halotolerant ammonia oxidizers in the beta-subclass of <i>Proteobacteria</i>	Wagner <i>et al.</i> , 1995
Nsv443	<i>Nitrosospira</i> spp.	Mobarry <i>et al.</i> , 1996
NmV	<i>Nitrosococcus mobilis</i> lineage	PommereningRoser <i>et al.</i> , 1996
NmII	Many members of the <i>Nitrosomonas communis</i> lineage	PommereningRoser <i>et al.</i> , 1996
Nmo218	Many members of the <i>Nitrosomonas oligotropha</i> lineage	Gieseke <i>et al.</i> , 2001
Nso1225	All known ammonia oxidizers in the beta-subclass except <i>N. mobilis</i>	Mobarry <i>et al.</i> , 1996

^a used with unlabeled competitor as indicated in the reference

All samples hybridized with oligonucleotide probes were embedded in Citifluor (Citifluor, Canterbury, United Kingdom) prior to microscopic observation. Samples for the analysis of time series were analyzed by standard epifluorescence microscopy on an Olympus BX50 microscope, equipped with filters HQ-CY3 and HQ-FLUOS (both from Analysentechnik AG, Tübingen, Germany). Quantification of nitrifying bacteria was carried out according to Manser *et al.* (2005c). For every sample and probe, 12 randomly chosen microscopic fields were analyzed.

Images for the spatial analysis of the flocs were recorded using a TCS NT confocal laser scanning microscope (Leica Microsystems, Bensheim, Germany) equipped with one Argon laser (450 to 514 nm) and two Helium-Neon lasers (543 and 633 nm). Three-dimensional reconstructions of flocs were generated from stacked thin optical sections.

2.3 Batch experiments and analytical measurements

Maximum nitrification rates were measured by respirometry in batch reactors with 7.5 liter volume as described by many authors (e.g. Cech *et al.*, 1985). The pH value was controlled and set to 7.5 in all experiments using HCl and NaOH. Temperature decreased from 23 to 17°C between the first and last experiment. Initial concentrations of ammonia were between 6 and 8 g_{NH₄-N} m⁻³.

The nitrification capacity of the biofilm on the membrane surface was assessed by taking out one module five weeks after chemical cleaning and immersing it into a cylindrical vessel filled with tap water and equipped with an air diffuser. Oxygen concentration was about

$8 \text{ g}_{\text{O}_2} \text{ m}^{-3}$. The initial concentration of ammonia was set to $10 \text{ g}_{\text{NH}_4\text{-N}} \text{ m}^{-3}$ by adding ammonia stock solution. Samples of the bulk medium were regularly analyzed for nitrate to calculate the nitrification rate.

The wastewater composition was analyzed for TSS, total COD, soluble COD, NH_4^+ , NO_3^- , NO_2^- , total N, PO_4^{2-} and total P. The effluents of both plants were analyzed for NH_4^+ , NO_3^- , NO_2^- , PO_4^{2-} , soluble COD (only MBR) and DOC, alkalinity and TSS (only CAS). 24h-composite samples were taken two to three times a week. Grab samples from the mixed liquors were analyzed twice a week for TSS, total COD, total N and total P. All analyses were performed according to *Standard Methods* (American Public Health Association, 1992).

3. Results and Discussion

3.1 Performance of the MBR and CAS

Table 2 summarizes the effectiveness of the MBR and the CAS system in the treatment of domestic wastewater. Excellent effluent quality was obtained in both systems. Approximately 90% of the total COD were removed in the CAS, whereas the removal efficiency of the MBR was 94%. The higher value of the MBR is mostly due to the complete retention of particulate matter. Both plants exhibited complete nitrification. Nitrite concentrations in the effluent of the CAS exhibited a more pronounced variation and were slightly higher. Further nitrification within the pores of the membrane or the increased air supply due to the coarse bubble aeration are possible reasons for the more stable nitrite oxidation in the MBR. Furthermore, the smaller floc size in the MBR compared to the CAS (data not shown) probably reduced the influence of mass transfer effects within the flocs. Removal efficiency of nitrogen was approximately 67% and 74% for the CAS and MBR system, respectively, assuming similar content of organic nitrogen in the permeate as in the CAS effluent.

The mixed liquor properties of the MBR and the CAS system are listed in Table 3. Due to the direct separation of solids from the treated wastewater, the total suspended solids (TSS) concentration in the aerobic tank of the MBR system is higher than in the anoxic tank, depending on the recirculation flow rate. Equal values for both activated sludges were found for the content of organics, nitrogen and phosphorus.

Table 2 Summary of the performance of the two pilot plants. Average values and standard deviations over a steady-state period of five months are shown.

Parameter	Unit	Influent	CAS effluent	MBR effluent
TSS	g m^{-3}	97 ± 31	8.0 ± 2.9	
Total COD	g m^{-3}	326 ± 102	32 ± 4^a	
Soluble COD	g m^{-3}	148 ± 66	n.d. ^c	19 ± 6
DOC	$\text{g}_C \text{ m}^{-3}$	n.d. ^c	8 ± 1	n.d. ^c
NH_4^+	$\text{g}_N \text{ m}^{-3}$	16 ± 5	0.16 ± 0.14	0.16 ± 0.09
NO_3^-	$\text{g}_N \text{ m}^{-3}$	0.8 ± 0.6	6.4 ± 2.3	5.0 ± 1.3
NO_2^-	$\text{g}_N \text{ m}^{-3}$	0.5 ± 0.4	0.21 ± 0.17	0.17 ± 0.05
Total N	$\text{g}_N \text{ m}^{-3}$	31 ± 6		
Total soluble N	$\text{g}_N \text{ m}^{-3}$	n.d. ^c	9.5 ± 2.2	n.d. ^c
PO_4^{2-}	$\text{g}_P \text{ m}^{-3}$	1.8 ± 0.6	1.6 ± 1.1	1.1 ± 0.7
Total P	$\text{g}_P \text{ m}^{-3}$	4.0 ± 1.0	1.7 ± 1.2^b	
Alkalinity	mol m^{-3}		7.3 ± 1.2	n.d. ^c

^a Calculated as sum of soluble COD ($\approx 3 \cdot \text{DOC}$) and COD content of TSS^b Calculated as sum of phosphate and P content of TSS^c not determined**Table 3** Mixed liquor properties of the two pilot plants. Average values and standard deviations over a steady-state period of five months are shown.

Parameter	Unit	CAS	MBR
TSS	g m^{-3}	3650 ± 844	4730 ± 573 (aerobic) 2980 ± 473 (anoxic)
i_{COD}	$\text{g}_{\text{COD}} \text{ g}_{\text{TSS}}^{-1}$	1.09 ± 0.15	1.09 ± 0.06
i_P	$\text{g}_P \text{ g}_{\text{COD}}^{-1}$	0.022 ± 0.005	0.022 ± 0.004
i_N	$\text{g}_N \text{ g}_{\text{COD}}^{-1}$	0.065 ± 0.011	0.069 ± 0.008

3.2 Nitrifying population

The evolution of the nitrifying bacteria shows a similar pattern for both CAS and MBR (Figure 2). The heterogeneous population of **ammonia-oxidizing bacteria (AOB)** is dominated by members related to the *Nitrosomonas oligotropha* lineage (Nmo218), which made up approximately 80% of all detected AOB. The smaller fraction consisted of members of the *Nitrosomonas europaea/eutropha* (NEU) and the *Nitrosomonas communis* lineage (NmII). Both were present throughout the whole period and accounted for 5% and 15% of all AOB, respectively. Members of the *N. oligotropha* lineage show high substrate affinity compared to *N. europaea/eutropha*-like and *N. communis*-like AOB and possess urease activity (Koops and Pommerening-Roser, 2001). Since ammonia concentrations are generally low in wastewater treatment plants (WWTPs) treating domestic wastewater, the prevailing conditions are advantageous for *N. oligotropha*-like AOB. However, the predominance of *N. oligotropha*-

like AOB is contrasting previous investigations of WWTPs, where members related to the *Nitrosomonas europaea/eutropha* lineage, the *Nitrosococcus mobilis* lineage and the *Nitrosomonas marina* cluster were most frequently detected (reviewed by Wagner *et al.*, 2002). Unfortunately, it is not reported whether most of these studies were carried out on WWTPs operated at low sludge ages, which would favour the above specified AOB. The high variation of the influent load probably provides niches for *N. europaea/eutropha*-like and *N. communis*-like AOB to occupy. But the low number of AOB detected by probe NEU during the investigated period possibly was also caused by inoculation from the sewer. The application of FISH to a raw wastewater sample failed because of the high fraction of dirt causing interfering signals under the microscope. Members of the *N. communis* lineage increased significantly in both MBR and CAS after circa 150 days of operation. The more diverse community composition after 150 days of operation did not influence the nitrification performance, but possibly enhanced the stability of the process due to functional redundancy (Daims *et al.*, 2001b). No signals were detected after hybridizations with probes NmV and Nsv443, indicating that neither members related to the *Nitrospira* group nor members related to *Nitrosococcus mobilis* could accumulate in the plants. This result is consistent with the hypothesis that generally nitrosomonads are responsible for ammonia oxidation in WWTPs and that nitrospiras occur only sporadically in these systems (Purkhold *et al.*, 2000). Hybridization with probe Nso1225 confirmed that no further hitherto known AOB were present in significant number.

Nitrite-oxidizing bacteria (NOB) belonged to the genus *Nitrospira* (Ntspa662) in both MBR and CAS, but could be detected only after about 120 days of complete nitrification (Figure 2). Since no members related to the genus *Nitrobacter* (NIT3) were found in any samples, it is supposed that either unknown nitrite-oxidizing bacteria were present in the first period or the rRNA content of the cells was below the detection limit for FISH. Alternatively, hybridization with probe Ntspa712 targeting the phylum *Nitrospira* did not reveal the presence of further NOB (data not shown). Thus, the low nitrite concentrations in the reactors favor the adaptation of K-strategists (high affinity to the substrate, low maximum growth rates) as *Nitrospira*-like NOB, while *Nitrobacter*-like NOB are postulated to be a relatively fast-growing r-strategist with low affinity to nitrite (Schramm *et al.*, 1999).

2. MBR vs. CAS - Population dynamics of nitrifiers

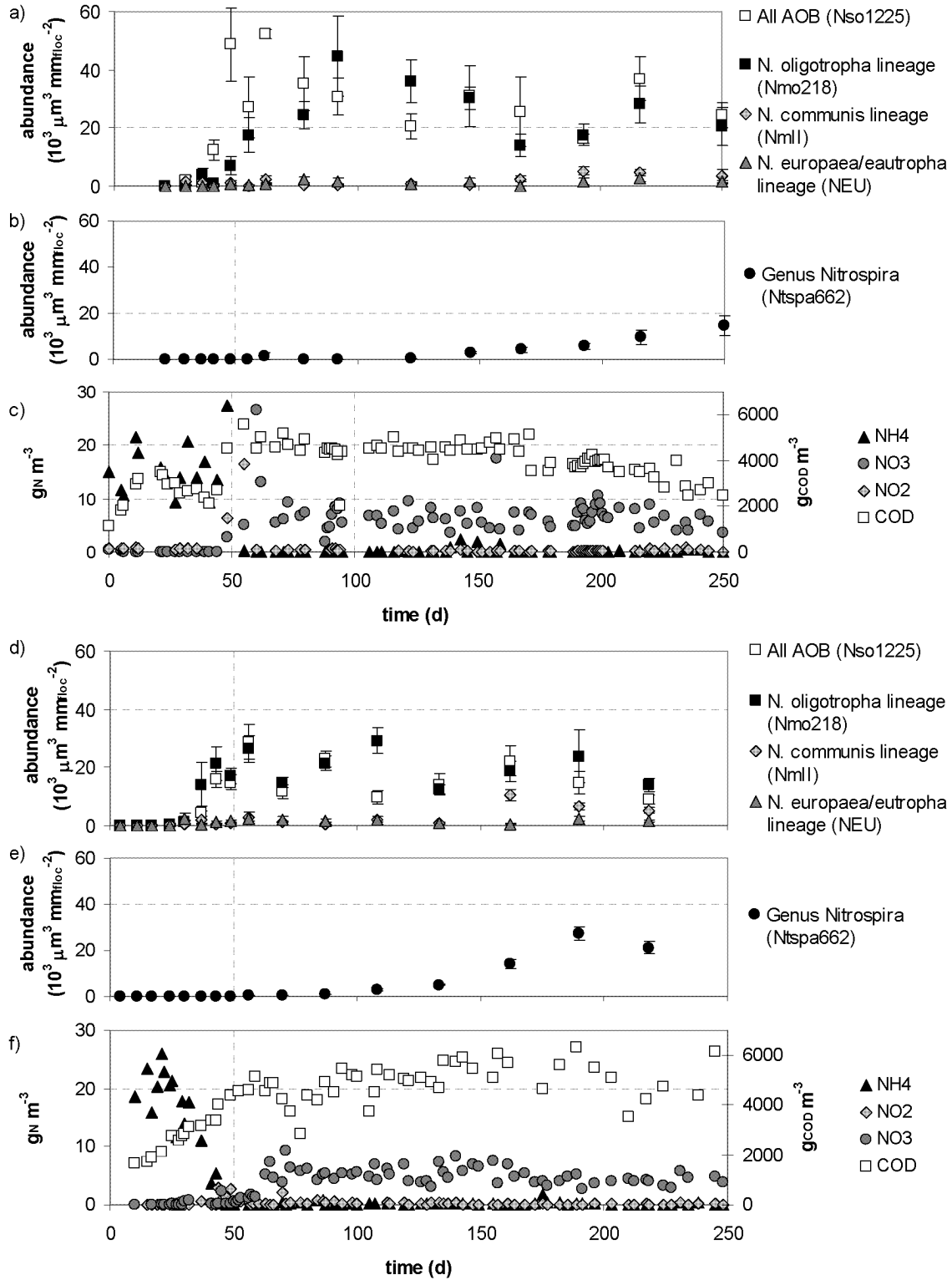


Figure 2 Time series of ammonia- (a, d) and nitrite-oxidizing bacteria (b, e) and concentration profiles (c, f) in the CAS (a-c) and MBR (d-f). Error bars indicate one standard deviation. Ammonia, nitrate and nitrite concentrations refer to the effluent of the plants, COD concentration to the activated sludge.

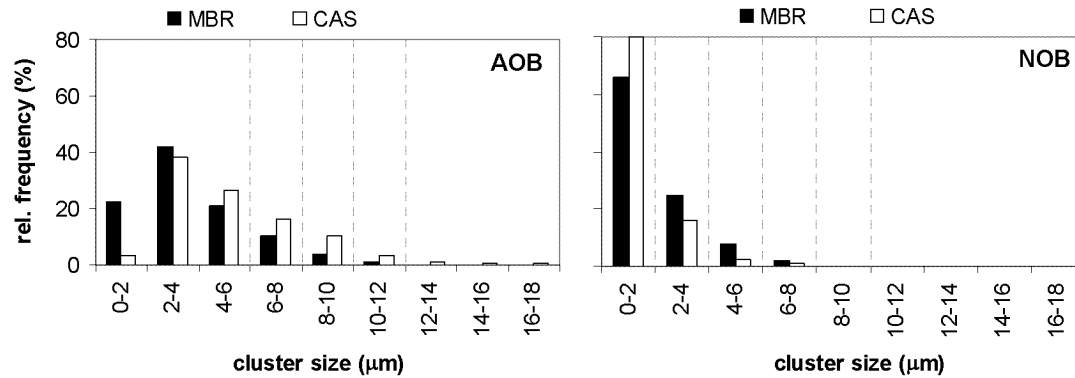


Figure 3 Cluster (=aggregate) size distribution of ammonia- (probe Nmo218) and nitrite-oxidizing bacteria (probe Ntspa662). Between 350 and 1400 cluster were counted for each distribution over a steady-state period of five months. Single cells were excluded.

Both AOB and NOB preferably grew in dense clusters. Generally the aggregates formed by NOB were smaller than those of ammonia-oxidizing groups (Figure 3). Daims *et al.* (2001a) found similar average diameters of *Nitrospira* clusters in conventional full-scale activated sludge plants. While AOB clusters up to diameters of 18 μm were present in the CAS system, the largest cluster size in the MBR was 12 μm. Similarly to the floc size, the high shear forces within the MBR possibly lead to the development of smaller aggregates. As a consequence, only 15% of all detected AOB in the MBR (Nmo218) were located in clusters larger than 10 μm, whereas it was 35% in the CAS system.

The spatial distribution of the nitrifiers within the activated sludge floc is crucial in terms of substrate and oxygen availability. Although the flocs were rather compressed on the slide due to the hybridization procedure, confocal laser scanning microscopy showed that the aggregates were located mostly in the inner part of the flocs (Figure 4). Most of the bacteria detected only by probe EUB338mix were considered to be heterotrophic bacteria, which overgrew the slow-growing nitrifiers. This observation suggests a potential oxygen limitation of nitrifiers in large flocs.

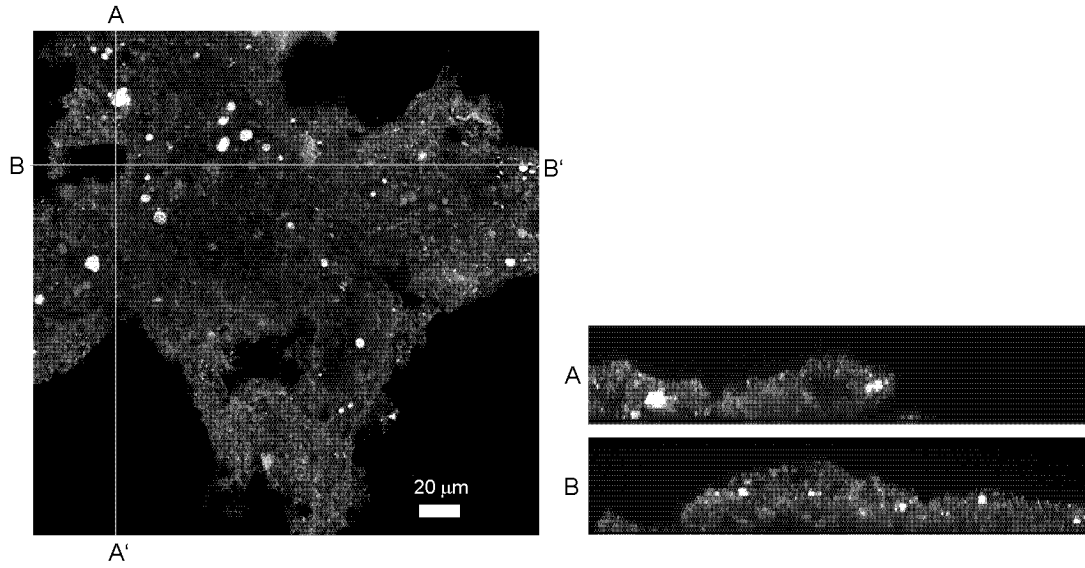


Figure 4 Analysis of the spatial distribution of AOB within an activated sludge floc from the CAS plant by confocal laser scanning microscope (CLSM). The distance between the horizontal layers is 2 µm. The white color shows AOB detected by both the Nmo218 probe and EUB338mix probe. The grey color shows other bacteria detected only by EUB338mix.

3.3 Nitrification rates

Both the CAS and the MBR system exhibited a similar maximum nitrification rate with average values of 33 ± 5 and 36 ± 5 $\text{mg}_\text{N} \text{g}_{\text{COD}}^{-1} \text{d}^{-1}$ at 20 °C, respectively (Figure 5).

First, at unlimited conditions with respect to substrate and oxygen concentrations, the same nitrifying community composition led to the same nitrification performance in both systems. However, the smaller floc size in the MBR compared to the CAS (data not shown), accentuated by the location of the nitrifiers in the inner part of the flocs, possibly lead to smaller mass transfer effects at limited conditions in MBR systems.

Secondly, knowing the maximum nitrification rates and nitrogen balance, the safety factor (SF) in terms of overloading can be estimated applying the following formula:

$$\text{SF}_{\text{overloading}} = \frac{\Delta L_{\text{Nit,max}}}{\Delta L_{\text{Nit,ave}}}$$

with: $\Delta L_{\text{Nit,max}}$ = maximum ammonium load that can be nitrified ($\text{g}_\text{N} \text{d}^{-1}$)

$\Delta L_{\text{Nit,ave}}$ = average ammonium load that is nitrified (CAS: 349, MBR: 11) ($\text{g}_\text{N} \text{d}^{-1}$)

The calculation yielded 2.7 and 2.6 for the CAS and the MBR, respectively. Furthermore, assuming typical maximum growth rates (1 d^{-1} at 20 °C; Gujer *et al.*, 1999) and aerobic decay constants (0.2 d^{-1} at 20 °C; Siegrist *et al.*, 1999), a good agreement with the calculated safety factors was found.

Since the membrane surface provides large areas for potential biofilm growth, the nitrification capacity of these biofilms was investigated. Surprisingly, the measured rate was

$15 \pm 2.5 \text{ mg}_\text{N} \text{ m}^{-2} \text{ d}^{-1}$, which is far lower than for typical nitrifying biofilms ($2000 - 3000 \text{ mg}_\text{N} \text{ m}^{-2} \text{ d}^{-1}$ at 15°C ; Boller *et al.*, 1990). Its contribution to the overall nitrification performance is negligible since the average nitrification rate of the reactor was about $4000 \text{ mg}_\text{N} \text{ m}^{-2} \text{ d}^{-1}$ in respect to the membrane surface ($8 \text{ l h}^{-1} \text{ m}^{-2}$ average hydraulic load per module). Thus, it is supposed that the shear stress of the coarse bubble aeration and the cyclic backwash procedure efficiently prevent the growth of a stable biofilm on the membrane surface.

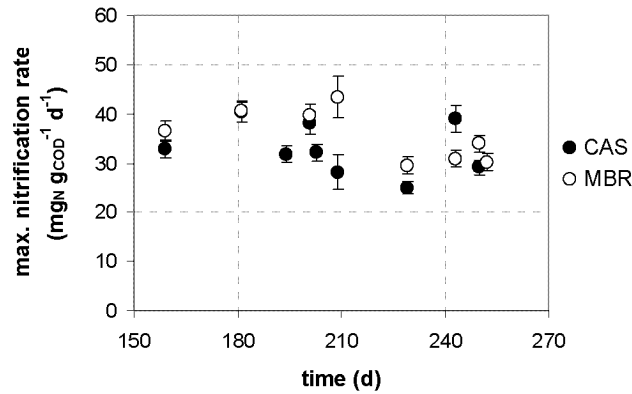


Figure 5 Overall maximum nitrification rates. Values given are corrected for temperature at 20°C . Error bars indicate one standard deviation.

4. Conclusions

The impact of the membrane separation on the nitrifying community composition and the performance of the process was investigated by running a MBR and CAS pilot in parallel treating the same wastewater. It is concluded from the experiments that

- the membrane separation itself does not influence the nitrifying community composition. The composition and heterogeneity of the AOB population and its associated stability of the process seem to be dependent on other factors like wastewater composition and sludge age. It is hypothesized that this finding can also be assigned to other groups within the activated sludge population.
- the membrane separation does neither enhance the nitrification performance nor improve the safety against overloading or wash-out.
- although the membranes provide a large surface area for potential biofilm growth, its contribution to the overall nitrification rate can be neglected.

Consequently, existing activated sludge models are valid also for MBR systems in terms of the model structure, but the characterization of the kinetics needs further research. The smaller floc size in the MBR compared to the CAS, accentuated by the location of the nitrifiers in the inner part of the flocs, possibly lead to smaller mass transfer effects in MBR systems.

CHAPTER 3

Bioaugmentation of Nitrifying Bacteria

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Manuscript in preparation

Bioaugmentation of Nitrifying Bacteria

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Abstract

Bioaugmentation is often suggested as a concept to improve the nitrification performance without the need of enlarging the aeration tanks. Furthermore, the increased application of separate treatment of digester liquids associated with centralized sludge digestion provides highly enriched nitrifying sludge, which could be used for seeding. This study investigated the potential of bioaugmentation using sludge from the separate treatment of digester liquids. It was found that different populations of nitrifying bacteria were present in the enriched sludge (dominated by *Nitrosomonas europaea* and *Nitrobacter*) compared to the activated sludge of a conventional and two membrane bioreactor pilot plants (dominated by *Nitrosomonas oligotropha* and *Nitrospira*) as detected by fluorescent in situ hybridization (FISH). The former exhibited a low substrate affinity and were not able to adapt to low substrate levels within days. As a consequence, the nitrifiers added to the fully nitrifying pilot plants were washed out without contributing to the conversion of ammonia and nitrite. By means of simulation studies, the effect of continuously adding sludge from the separate treatment of digester liquids to wastewater treatment plants was investigated. The results indicate that the enriched nitrifying sludge is not suitable to enhance the nitrification performance of municipal wastewater treatment plants.

Keywords: Activated sludge; Bioaugmentation; FISH; Kinetics; Membrane Bioreactor; Modeling; Nitrification; Population dynamics

1. Introduction

Historically, only COD was removed in wastewater treatment plants (WWTPs), involving relatively small tank volumes. Nowadays, stricter effluent regulations especially in terms of N-removal have to be met. Nitrifying bacteria have low growth rates in comparison to heterotrophic bacteria, which results in large volumes of aeration tanks to guarantee all-season nitrification. Denitrification needs often to be achieved too, leading to even larger tank volumes. In addition, the separate treatment of sludge digester liquids indeed decreases the overall nitrogen load, but increases the diurnal variation of the N-Kj (total Kjeldahl nitrogen) load, which augments the required aerobic sludge retention time (SRT).

Bioaugmentation was introduced as a concept to improve the nitrification performance without the need of enlarging the aeration tanks. Accumulation of nitrifying bacteria used for addition is carried out in a separate reactor either outside of the activated sludge stream (Head and Oleszkiewicz, 2004; Plaza *et al.*, 2001) or within the return sludge line (Salem *et al.*, 2003). Both systems are typically seeded with ammonium-rich liquids originating e.g. from sludge dewatering. The main advantage of the latter system is the enrichment of already adapted nitrifiers, whereas different type of nitrifying bacteria seems to be present in processes with high influent ammonium concentrations, e.g. the SHARON[®] process (Logemann *et al.*, 1998).

However, it is not clear whether nitrifiers grown at high ammonium concentrations can adapt to low substrate concentrations prevailing in municipal WWTPs. In addition, membrane bioreactors (MBR) exhibit a different sludge structure compared to conventional activated sludge plants (CAS, Manser *et al.*, 2005a) and completely retain bacteria, which prevents loss of seeded bacteria due to insufficient settleability.

The goal of this study was to investigate the potential of bioaugmentation using enriched nitrifying bacteria from the separate treatment of digester liquid. This included the analysis of the autotrophic community compositions, measurement of their kinetics and examination of the competition among the different groups. Furthermore, the potential positive effect of membrane separation on the bioaugmentation performance was explored. Fluorescent in situ hybridization (FISH) was applied for tracking population dynamics. Batch experiments provided kinetic data.

2. Materials and Methods

2.1 Pilot plants

A CAS and two identical MBR pilot plants (Manser *et al.*, 2004) were operated in parallel treating municipal wastewater following a typical diurnal hydraulic variation. The CAS and the MBRs treated the wastewater corresponding to 60 ($18 \text{ m}^3 \text{ d}^{-1}$) and 2 ($0.56 \text{ m}^3 \text{ d}^{-1}$) population equivalents, respectively. Oxygen concentration in the aerobic tanks was controlled between 2.5 and 3 g m^{-3} . Due to the coarse bubble aeration inducing a cross-flow at the membrane surface, the oxygen concentration was temporarily higher in the MBRs. Both plants were operated with pre-denitrification and the sludge retention time (SRT) was kept at 20 days for the CAS and MBR 1 and 40 days for the MBR 2.

A sequencing batch reactor (SBR, volume 3 m^3) was inoculated with sludge from the SBR for the separate treatment of digester liquid at the WWTP of Bülach (Switzerland), fed with a synthetic ammonia solution and continuously aerated. The pH value was controlled using NaCO_3 and set to 7.5 ± 0.1 . The SBR was operated twice for about two weeks. Each period was started with inoculum from the WWTP of Bülach. Community compositions did not change over this period.

2.2 Batch experiments

A) Half-saturation constants for substrate (ammonia, nitrite). Activated sludge was aerated in batch reactors with 7.5 liter volume for at least two hours prior to adding the substrate to ensure that all biodegradable matters were consumed. The temperature and pH value was controlled and set to $15.0 \pm 0.2^\circ\text{C}$ and 7.50 ± 0.05 using HCl or NaOH in all experiments, respectively. Oxygen concentration was controlled between 3 and 4 g m^{-3} in order to prevent oxygen limitations. AOB were inhibited by 10 g m^{-3} allylthiourea (K_s for nitrite only). Samples were taken regularly until all substrate was consumed. Substrate concentrations of the samples were

measured according to *Standard Methods* (American Public Health Association, 1992). Additionally, oxygen utilization rate (OUR) data from experiments for the determination of the maximum nitrification activity (B) were used for the estimation of the K values.

B) Maximum nitrification activity. Nitritation and nitrataion rates were measured by means of respirometry (Drtil et al., 1993) in a batch reactor (Volume 7.5 liter). The pH value was controlled and set to 7.50 ± 0.05 in all experiments. The temperature in the batch reactors was kept constant (15 or 17°C) during the experiments. Added ammonium and nitrite concentrations were: $100 \text{ g}_{\text{NH}_4\text{-N}} \text{ m}^{-3} / 20 \text{ g}_{\text{NO}_2\text{-N}} \text{ m}^{-3}$, $10 \text{ g}_{\text{NH}_4\text{-N}} \text{ m}^{-3} / 4 \text{ g}_{\text{NO}_2\text{-N}} \text{ m}^{-3}$ and $5 \text{ g}_{\text{NH}_4\text{-N}} \text{ m}^{-3} / 2 \text{ g}_{\text{NO}_2\text{-N}} \text{ m}^{-3}$ for sludge mixtures; $100 \text{ g}_{\text{NH}_4\text{-N}} \text{ m}^{-3} / 20 \text{ g}_{\text{NO}_2\text{-N}} \text{ m}^{-3}$ for the bioaugmentation experiment (Chapter 2.3); $500 \text{ g}_{\text{NH}_4\text{-N}} \text{ m}^{-3} / 75 \text{ g}_{\text{NO}_2\text{-N}} \text{ m}^{-3}$ for the decay experiment (C).

C) Autotrophic decay. A batch reactor (130 liter) was filled with sludge from the SBR pilot reactor and continuously aerated. The pH value was controlled and set to 7.5 ± 0.1 . The temperature was kept at 15°C. At intervals of one day, 7.5 liter of activated sludge was withdrawn from the batch reactor and placed in a secondary batch reactor, where nitritation and nitrataion rates were measured by respirometry. Nitrite-oxidizing bacteria are probably inhibited by the elevated nitrate concentrations. Batch experiments revealed that a sudden increase of the nitrate concentration from 60 to 300 $\text{g}_{\text{N}} \text{ m}^{-3}$ reduced the maximum nitrataion rates by 50%. However, a constant inhibition can be assumed during the experiment, since the nitrate concentrations only slightly increased during the experiment.

2.3 Bioaugmentation experiment

Large amounts of SBR sludge were once-only added to the anoxic tanks of the CAS (2.7 m^3) and MBRs (0.05 m^3 each). The spiking of the CAS took about one hour, whereas the MBRs was instantaneously seeded.

2.4 Fluorescence *In Situ* Hybridization (FISH)

Samples were taken at maximum OUR and fixed in 4% paraformaldehyde as described by Amann *et al.* (1990). Ultrasonification (35 kHz, five minutes) was applied to fixed samples from the CAS prior to hybridization in order to break up large flocs. *In situ* hybridizations of cells were performed with fluorescently labeled, rRNA-targeted oligonucleotide probes according to Manz *et al.* (1992) (Table 1). Oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland) and Thermo Hybaid (Interactiva Division, Ulm, Germany).

All samples hybridized with oligonucleotide probes were embedded in Citifluor (Citifluor, Canterbury, United Kingdom) prior to microscopic observation. Epifluorescence microscopy was done on an Olympus BX50 microscope, equipped with filters HQ-CY3 and HQ-FLUOS (both from Analysentechnik AG, Tübingen, Germany). Quantification of nitrifying bacteria was carried out according to Manser *et al.* (2005c). 14 microscopic fields were analyzed per sample and probe.

Table 1 Oligonucleotide probes and hybridization conditions applied in this study.

Probe	Target organisms	Reference
NEUa	Most halophilic and halotolerant ammonia oxidizers in the beta-subclass of Proteobacteria	Wagner et al., 1995
NmII	Many members of the <i>Nitrosomonas communis</i> lineage	Pommerening-Roser et al., 1996
Nmo218	Many members of the <i>Nitrosomonas oligotropha</i> lineage	Gieseke et al., 2001
Nit3 ^a	<i>Nitrobacter</i> spp.	Wagner et al., 1996
Ntspa662 ^a	Genus <i>Nitrospira</i>	Daims et al., 2001a

^a used with unlabeled competitor as indicated in the reference

2.5 Modeling

For a correct representation of the investigated processes, the activated sludge model No. 3 (ASM3, Gujer *et al.*, 1999) was extended with two-step nitrification to enable separate modeling of ammonia and nitrite oxidation. Denitrification of nitrite and from nitrate to nitrite was not included, because this study was focused on nitrification. Furthermore, two populations of AOB and NOB exhibiting distinct kinetic parameters were introduced (Table 3, see appendix). Kinetic data of the SBR were assigned to one (X_{AOBr} , X_{NOBr}), kinetic data of the CAS and MBRs to the second population (X_{AOBk} , X_{NOBk}).

3. Results and Discussion

3.1 Community composition of nitrifying bacteria

The analysis of the **ammonia-oxidizing bacteria (AOB)** community composition exhibited a clear difference between the SBR (fed with digester liquid) and the pilot plants (fed with municipal wastewater, Figure 1). The CAS and MBRs were dominated by members related to the *Nitrosomonas oligotropha* lineage (Probe Nmo218, white bars), which made up 70-80% of all detected AOB. These bacteria show a high substrate affinity and low maximum growth rates and are therefore well adapted to conditions prevailing in completely nitrifying plants (Koops and Pommerening-Roser, 2001; Prosser, 1989). By contrast, in the SBR reactor members of the *Nitrosomonas europaea/eutropha* (Probe NEU, black bars) were most abundant. This group is characterized by a low substrate affinity, high maximum growth rates and a high salt tolerance (Bollmann *et al.*, 2002; Koops and Pommerening-Roser, 2001), which is advantageous in environments with elevated ammonium concentrations. The third group made up approximately 20% in all reactors and consisted of members of the *Nitrosomonas communis* lineage (Probe NmII, grey bars). Their phylogenetic characteristics are between the *N. oligotropha*-like and *N. europaea*-like AOB.

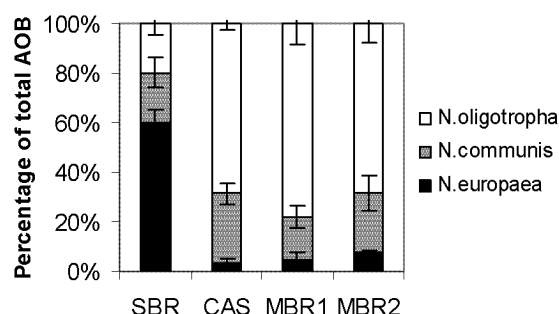


Figure 1 Population compositions of AOB before bioaugmentation. Error bars indicate \pm one standard deviation of each group of AOB ($n = 3$).

Nitrite-oxidizing bacteria (NOB) showed similar differences. The NOB in the CAS and MBRs belonged to the genus *Nitrospira* (Probe Ntspa662). They have a high affinity to the substrate and low maximum growth rates (Schramm *et al.*, 1999). Conversely, in the SBR members related to the genus *Nitrobacter* (Probe NIT3) - exhibiting a low affinity to the substrate and high maximum growth rates - were present. Unfortunately, problems with the hybridizations of probe NIT3 did not allow the quantification of further samples.

In other words, the SBR was dominated by r-strategists (low affinity to the substrate and high maximum growth rates; Andrews and Harris, 1986), whereas K-strategists (high affinity to the substrate and low maximum growth rates) were most abundant in the CAS and the MBRs.

3.2 Kinetic parameters

The reliable modeling of the co-existence of different bacteria populations using the same substrate requires detailed knowledge about the kinetic parameters. In particular, the half-saturation constants for the substrate (K_{NH_4} and K_{NO_2} of Monod-kinetics) and the net growth rate ($\mu_{max}-b$) are crucial, because they determine whether a certain bacterium is washed out or outcompeted by the better adapted groups under certain conditions. Therefore, the main focus was laid on the determination of the half-saturation constants and the maximum growth rates (μ_{max}) of AOB and NOB. Unfortunately, product inhibition effects adversely influenced the measurement of the latter impairing the estimation of the growth rates (data not shown).

Half-saturation constants (K_{NH_4} and K_{NO_2})

Half-saturation constants of SBR sludge were estimated from concentration profiles of ammonium and nitrite (Figure 2). Additionally, OUR data from different batch experiments with SBR, CAS and MBR sludge were used for the estimation of the K values (Figure 3).

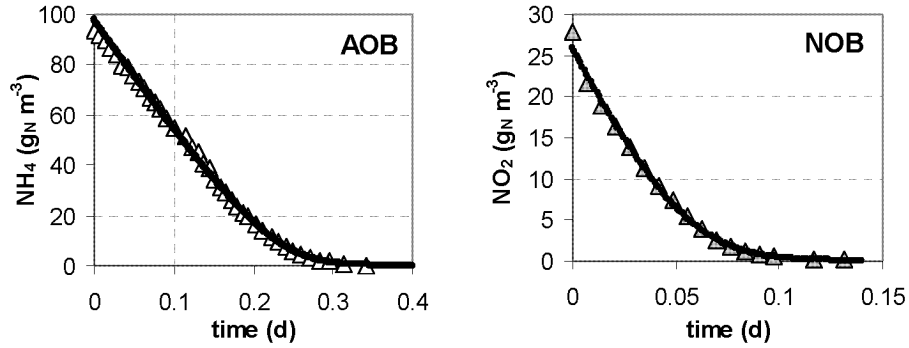


Figure 2 Measured concentration profiles of SBR sludge. Lines represent best fit to the extended ASM3.

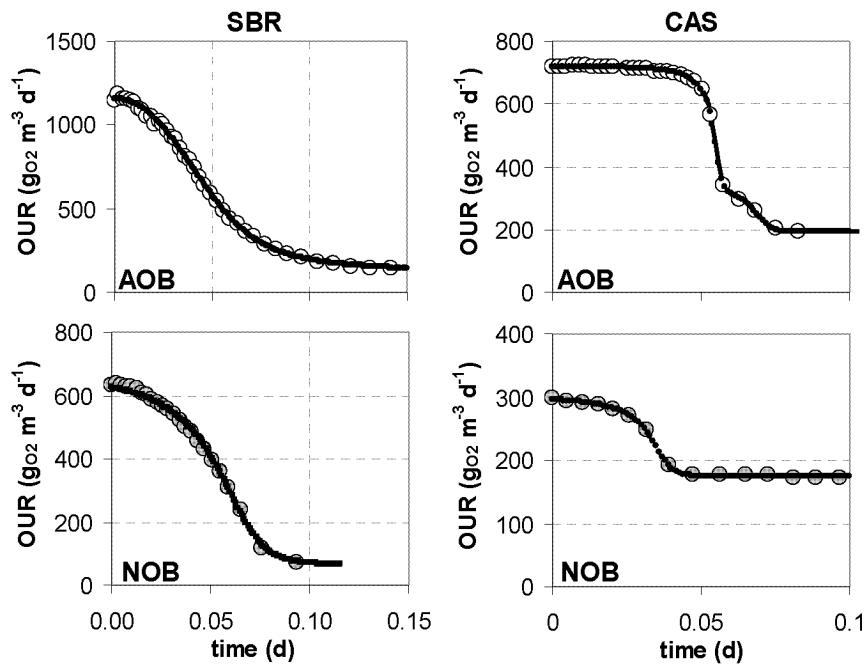


Figure 3 Measured and modeled OUR used for estimation of K_{NH_4} and K_{NO_2} values. Lines represent best fit to the extended ASM3. Upper diagrams: Nitrification due to produced nitrite lags behind nitrification (particularly visible in the CAS).

The estimation of the half-saturation constants of SBR sludge yielded high values for both AOB and NOB (Table 2), compared to low values in the CAS and MBR1. The obtained values from the OUR data correspond well with the values estimated from the concentration profiles for all examined sludges. Although the K_{NH_4} and K_{NO_2} values in the SBR vary considerably possibly due to inhibition effects (Chapter 2.2), it can be stated that the values in the SBR exceed those obtained from the CAS and MBR1 by one to two orders of magnitude. This kinetic observation is in agreement with the detected community compositions (Chapter 3.1).

Table 2 Estimated K_{NH_4} and K_{NO_2} from the measurement of the concentration profiles and the oxygen utilization rates (OUR). Given are standard errors of the estimations and standard deviations for single and mean values, respectively. A) estimated from concentration profiles, B) estimated from OUR profiles.

SBR		CAS		MBR1		
K_{NH_4} ($\text{g}_\text{N} \text{ m}^{-3}$)	K_{NO_2} ($\text{g}_\text{N} \text{ m}^{-3}$)	K_{NH_4} ($\text{g}_\text{N} \text{ m}^{-3}$)	K_{NO_2} ($\text{g}_\text{N} \text{ m}^{-3}$)	K_{NH_4} ($\text{g}_\text{N} \text{ m}^{-3}$)	K_{NO_2} ($\text{g}_\text{N} \text{ m}^{-3}$)	
9.9 ± 0.5	9.2 ± 1.1	$0.14 \pm 0.07^{1)}$	$0.28 \pm 0.20^{1)}$	$0.13 \pm 0.05^{1)}$	$0.17 \pm 0.06^{1)}$	A)
14.1 ± 0.6	2.9 ± 0.3					
6.0 ± 0.3	15.2 ± 2.3	0.11 ± 0.01	0.21 ± 0.02	0.1	0.21 ± 0.05	B)
	4.2 ± 3.0		0.24 ± 0.02			

¹⁾ mean value of three measurements (Manser *et al.*, 2005a)

Autotrophic decay

The net growth rate – defined as maximum growth rate minus decay – determines whether a bacterium can grow in an activated sludge system or is washed out at a given SRT. Therefore, knowledge about the decay of the nitrifiers used for bioaugmentation is needed in order to model their fate in the augmented plants. The estimation of the endogenous respiration rates of the SBR sludge yielded a significantly lower value for the AOB than for the NOB (Figure 4). The values converted to 20°C (temperature correction factors: $\theta_{\text{AOB}} = 0.105^\circ\text{C}^{-1}$, $\theta_{\text{NOB}} = 0.062^\circ\text{C}^{-1}$, Jenkins, 1969) are 0.22 d^{-1} and 0.31 d^{-1} for AOB and NOB, respectively. In comparison to endogenous respiration rates estimated from activated sludge, which was dominated by K-strategists (Manser *et al.*, 2005b), the same values here (sludge dominated by r-strategists) are about 50% higher. Interestingly, the AOB fractions showed distinct decay patterns as analyzed by FISH (Figure 4, middle). While the abundance of the *N. europaea*-like bacteria (black) sharply decreased, *N. communis*-like bacteria (grey) were less reduced and *N. oligotropha*-like bacteria (white) were even able to grow slightly. The latter group (K-strategists) probably outcompeted the other groups for the released nitrogen originating from microbial decay and could therefore maintain its presence, leading to a shift in the community composition of AOB.

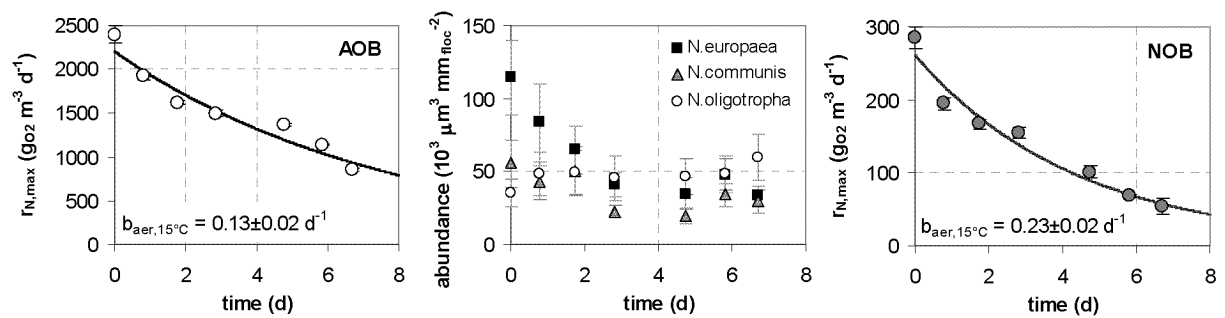


Figure 4 Decay of AOB (left) and NOB (right) from SBR sludge. T = 15°C, pH = 7.5. Middle: Quantitative FISH analysis of AOB. Error bars indicate one standard deviation (n = 14).

3.3 Batch experiments with sludge mixtures

Batch experiments with sludge mixtures were carried out in order to test whether the model reasonably reflects the measured activity associated with the co-existence of r-strategists (SBR, model parameters: X_{AOBr} , X_{NOBr}) and K-strategists (CAS, MBRs, model parameters: X_{AOBk} , X_{NOBk}). On the other hand, it is uncertain, whether r-strategists can rapidly adapt to conditions with low substrate concentrations. Therefore, maximum nitrification rates of sludge mixtures were determined at different substrate levels.

At **high substrate concentrations**, the measured rates of different mixtures of SBR and CAS sludge correspond well to the calculated sum of the individual rates, since both populations are not limited by the substrate (Figure 5). The fast-growing r-strategists from the SBR did not impair the slower-growing K-strategists from the CAS.

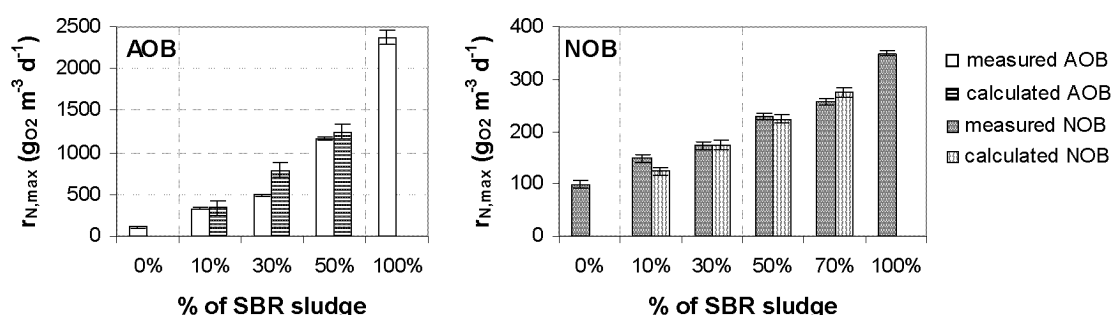


Figure 5 Measured and calculated OUR of mixtures of CAS and SBR sludge at high substrate concentrations (NH_4 : $100 \text{ g}_\text{N} \text{ m}^{-3}$, NO_2 : $20 \text{ g}_\text{N} \text{ m}^{-3}$). 0% of SBR sludge means 100% of CAS sludge. $T = 17^\circ\text{C}$, $\text{pH} = 7.5$. Error bars indicate \pm one standard deviation.

Likewise, the measured rates of mixtures of SBR and CAS sludge at **low substrate concentrations** are equal or slightly lower than the corresponding sum of the individual rates (Figure 6). Mixtures of SBR and MBR1 sludge yielded similar results (data not shown). The K-strategists (CAS, MBR) possibly interfere with the r-strategists (SBR) at low substrate levels. In contrast to high substrate concentrations, the r-strategists only reach 30-50% of their maximum activity due to significant substrate limitation. Obviously, they are not able to adapt to lower substrate concentrations in the short term (hours, Figure 6). As a consequence, they are poor competitors in WWTPs with full nitrification associated with low ammonium and nitrite concentrations.

Nonetheless, the addition of SBR sludge to the CAS and MBRs may contribute to the overall nitrification performance; if the indigenous nitrifying bacteria are washed out in winter (Figure 10). However, only partial nitrification can be achieved depending on the amount of added nitrifiers.

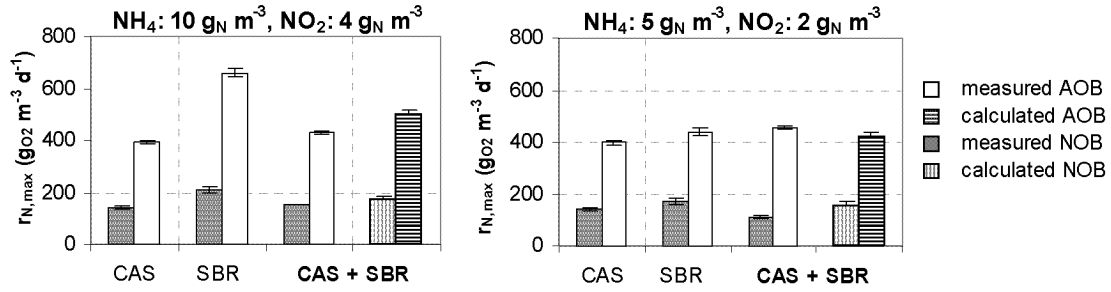


Figure 6 Measured and calculated OUR of mixtures of SBR (~ 50%) and CAS (~ 50%) sludge at low substrate concentrations. T = 15°C, pH = 7.5. Error bars indicate \pm one standard deviation.

Finally, the dynamic behavior of the sludge mixture was examined (Figure 7). The measurements at low substrate concentrations could be well reproduced with the parameters found from individual experiments (Table 2), indicating a parallel substrate uptake of the two populations according to their individual kinetic characteristics. The model shows that although the activity of both r- and K-strategists was equal at the beginning of the experiment, the contribution of the r-strategists to the overall OUR quickly declined, whereas the K-strategists were close to their maximum activity until almost all substrate was consumed.

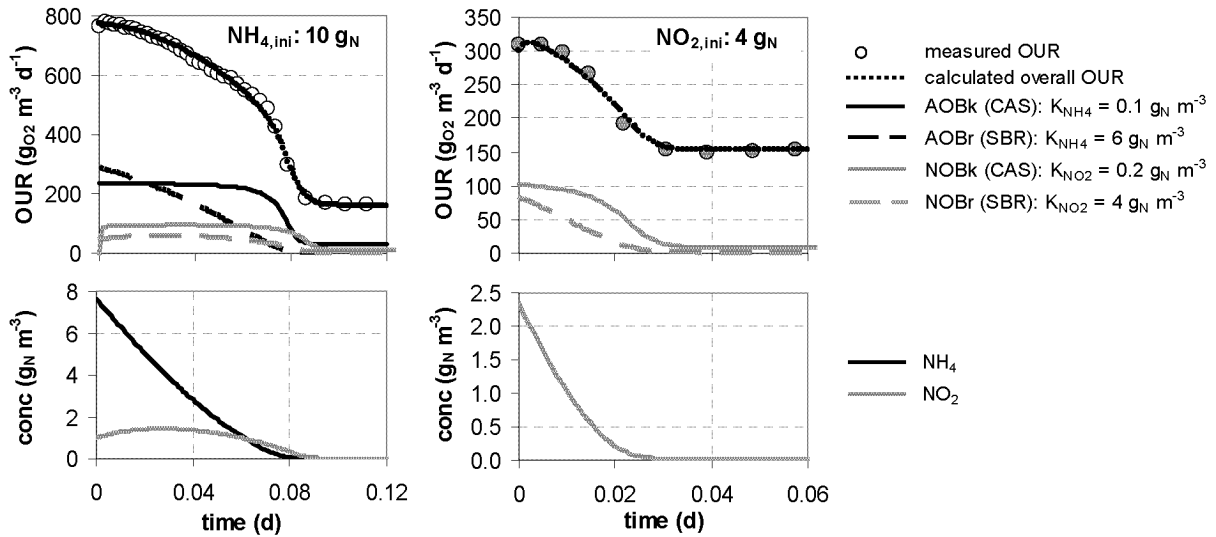


Figure 7 Measured and modeled OUR of mixtures of CAS and SBR sludge at low substrate concentrations.

3.4 Bioaugmentation

The influence of the addition of nitrifiers from the SBR on the **nitritation performance** and on the community composition of AOB in the CAS and MBRs is shown in Figure 8. It can be seen that the maximum nitritation rates as determined at non-limiting substrate concentrations in batch experiments sharply increased in all pilot plants after the addition of SBR sludge on day 14. The observed rise agreed well with the added amount of AOB. However, the aug-

mented activity exponentially decreased and reached the initial value after about 20 (CAS, MBR 1) and 30 days (MBR2). Although the quantification of the AOB was partly associated with large uncertainties due to the high spatial variability caused by the added sludge, valuable information was obtained. It was found that the added AOB (mostly *N. europaea*) were washed out simultaneously with the reduction of the maximum nitrification performance. Conversely, the AOB dominating in the pilot plants (*N. oligotropha*) were only slightly enriched by the addition and did not significantly alter during the experiment in all plants. The modeled nitrification rates show that the r-strategists (AOBr) were inactivated due to the low substrate concentrations and did not contribute to the nitrification in the pilot plants. Hence, the decrease of the modeled maximum nitrification rates and the biomass concentrations of X_{AOBr} only reflect decay and the dilution rates. The good agreement with the measured data suggests that the added AOB were not able to adapt to the conditions prevailing in the pilot plants within the period of the experiment, equally to the batch experiment for the short-term (Chapter 3.3). Otherwise, the observed decrease of the *N. europaea* should have been decelerated. The fact that accumulated bacteria may be inadequately protected against predation (Bouchez *et al.*, 2000) was not observed since it would have led to a sharper decrease of the added amount of nitrifiers. The organization of AOB and NOB as dense aggregates (Manser *et al.*, 2004; Wagner *et al.*, 1995) possibly prevent autotrophic bacteria from major predation.

All three pilot plants exhibited the same behavior. Neither the complete retention of single cells nor the different sludge structure in MBRs (Manser *et al.*, 2005a) seem to play a role for nitrifying bacteria. A larger SRT only delays the wash-out of the added AOB (MBR 2). Since the amount of nitrifying bacteria is directly dependent on the nitrified nitrogen load, the patterns of the modeled nitrification rates only reflect the temporal variability of the influent nitrogen load and are consequently identical for all three pilot plants.

3. Bioaugmentation of nitrifying bacteria

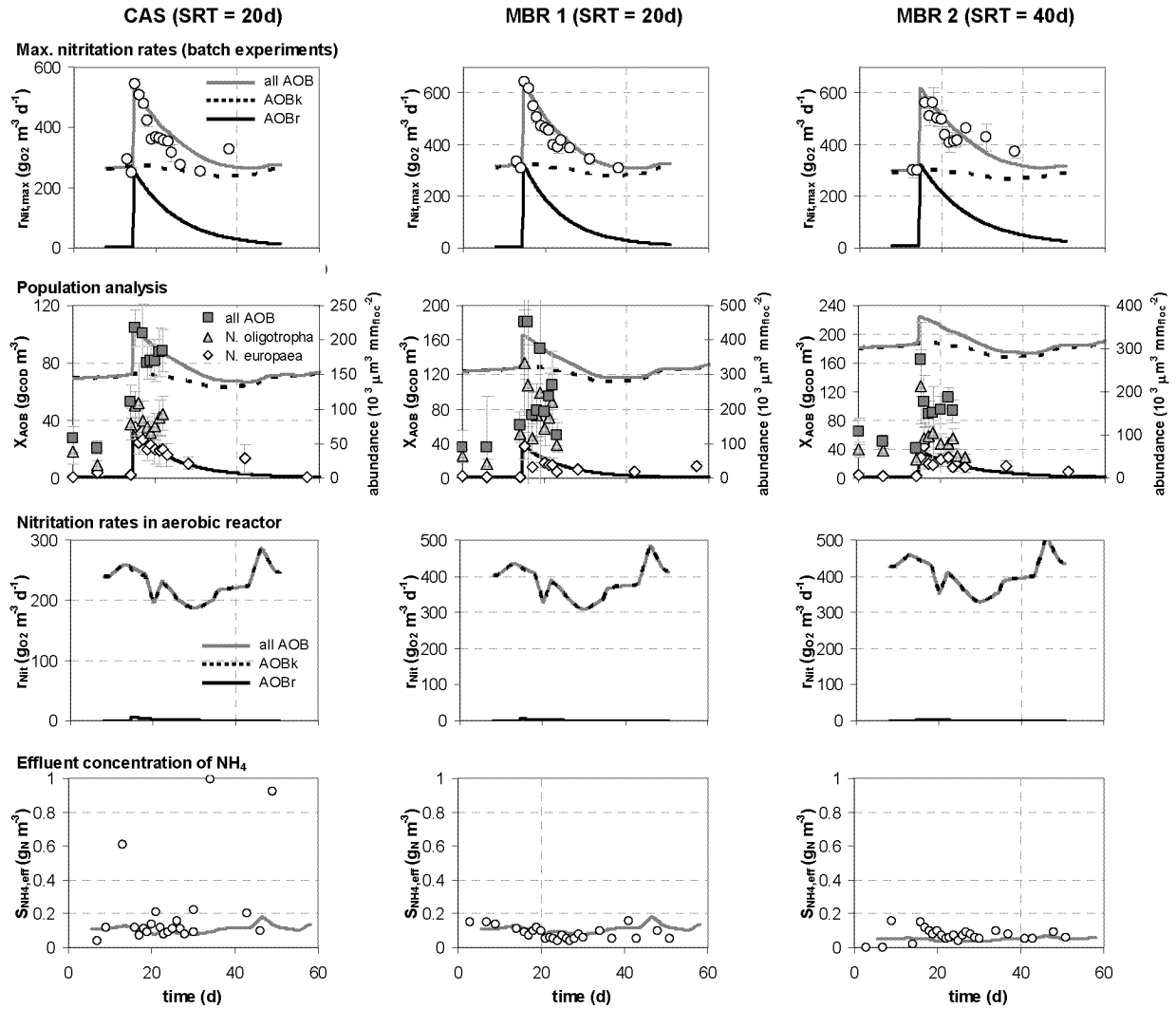


Figure 8 Measured and modelled data of AOB for bioaugmentation experiment. Error bars indicate \pm one standard deviation. SBR sludge was added on day 14.

The influence of the addition of nitrifiers from the SBR on the **nitrification performance** in the CAS and MBRs is shown in Figure 9. Analogous to the nitrification, the maximum nitrification rates as determined at non-limiting substrate concentrations in batch experiments sharply increased according to the added amount of NOB in all pilot plants, subsequently quickly decreased and reached the initial value after about 20 (CAS, MBR 1) and 30 days (MBR2). Unfortunately, problems with the hybridizations of the probe targeting *Nitrospira* (r-strategist) did not allow the analysis of the community composition. Nonetheless it is assumed that the decline of the maximum nitrification rates is only attributed to the wash-out of the added NOB (r-strategists), because the model suggests that their activity in the aerated tanks was negligible. Accordingly, the added NOB were equally to the AOB not able to adapt to low substrate concentrations.

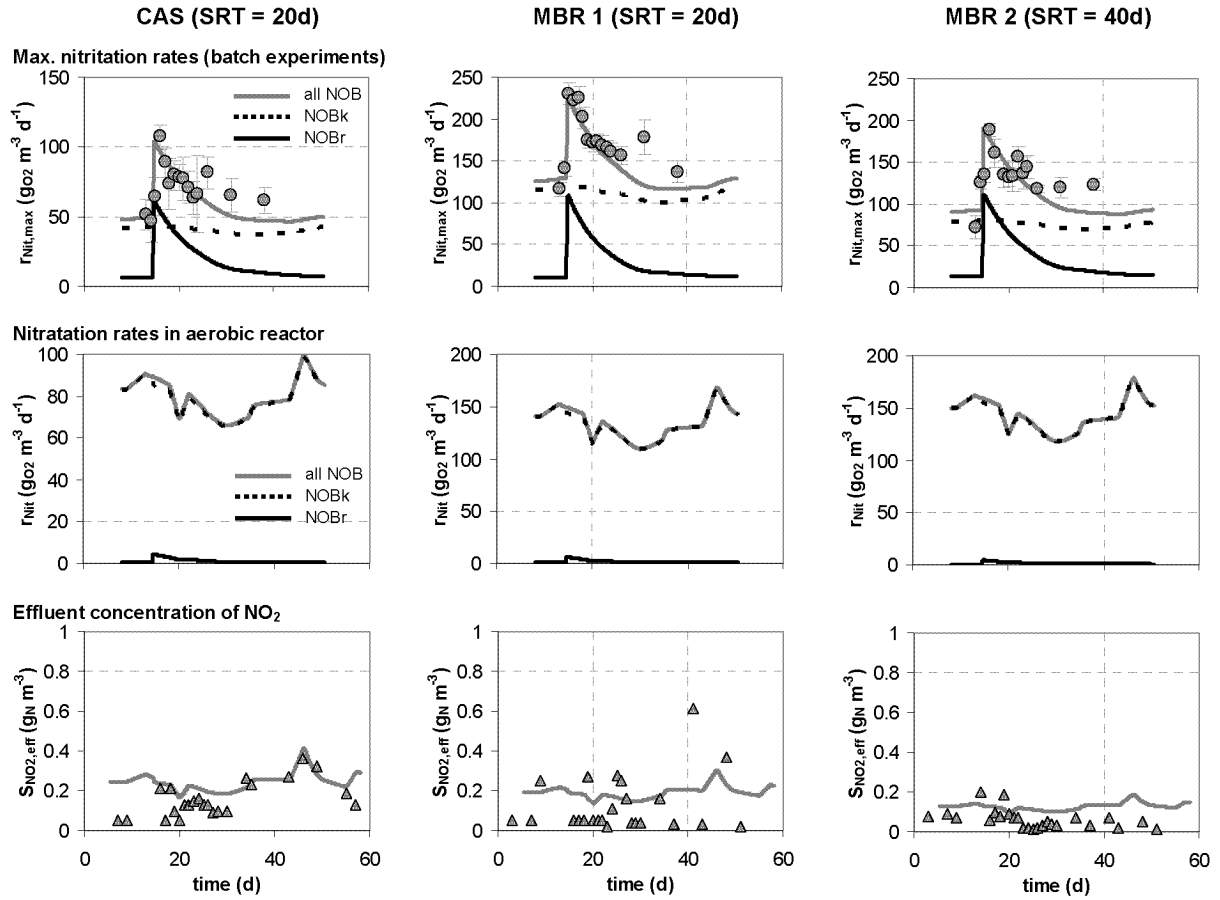


Figure 9 Measured and modeled data of NOB for bioaugmentation experiment. Error bars indicate \pm one standard deviation. SBR sludge was added on day 14.

3.5 Significance for wastewater treatment

The potential of bioaugmentation using enriched nitrifying sludge from the separate treatment of digester liquids was elucidated. Salem *et al.* (2003) showed that bioaugmentation is most beneficial when a system is operated somewhat below the minimal SRT required for nitrification. Therefore, simulations were performed assuming the following plant layout of a CAS: aerobic SRT = 4d, HRT = 6h, influent ammonium concentration = 25 gN m^{-3} and $T = 10^\circ\text{C}$. This plant would completely nitrify in summer season. It exhibits however no or only partial nitrification in winter season, because the minimal SRT required for all-season nitrification is higher than four days. The results of steady-state calculations are depicted in Figure 10. Dynamic calculations assuming a typical diurnal variation of municipal wastewater did not significantly change the results (data not shown). The daily amount of added nitrifiers is expressed in percent of the total input N-Kj load, which is returned in the digester liquid to the separate treatment and used for the enrichment of nitrifying bacteria. The internal feeding of ammonium is limited up to around 30% of the input N-Kj load (Salem *et al.*, 2003). The simulations reveal that the maximum addition (30% of the input N-Kj load) reduces the ammonium load in the effluent by almost 50% due to growth of the added r-strategists (AOBr) in

the CAS. The indigenous AOB population (AOBk) does not contribute to the nitrification performance. By contrast, the bioaugmentation leads to elevated nitrite concentrations in the effluent, because the added NOB population is not adapted to low nitrite levels. As a consequence, nitrite oxidation is mainly attributed to the K-strategists (NOBk), although the added r-strategists (NOBr) are equally abundant or even exceed the former at the maximum bioaugmentation dose. Nonetheless, The NOBk are not able to completely oxidize the produced nitrite because of its lower growth rates compared to the AOB. Moreover, the separate treatment of digester liquids takes place at elevated temperatures due to the warm influent. The enriched bacteria are therefore poorly adapted to the low temperatures in the basins. Hence, the simulated enhancement of the nitrification performance is possibly overestimated, since a sudden decrease in temperature may have a negative impact on nitrification rates (Abeyasinghe *et al.*, 2002; Head and Oleszkiewicz, 2004).

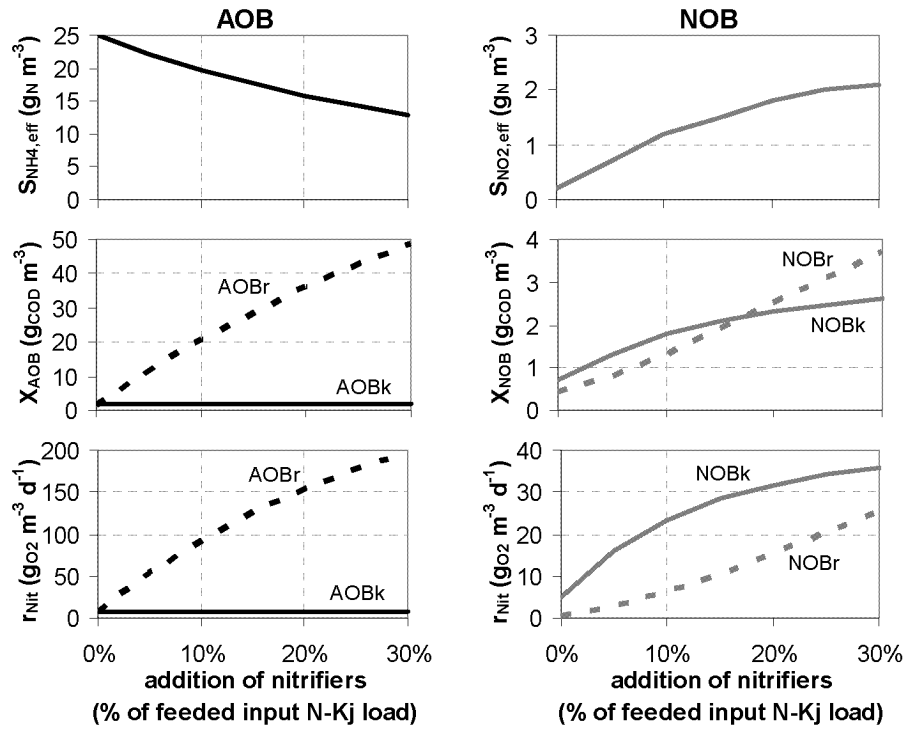


Figure 10 Simulation study for bioaugmentation assuming CAS plant layout with SRT = 4d, HRT = 6h, T = 10°C. As an example, 10% addition of nitrifiers means that 10% of the total input N-Kj load is returned in the digester liquid. Results of steady-state calculations are shown.

4. Conclusions

Bioaugmentation of a conventional activated sludge and two membrane bioreactor pilot plants using enriched nitrifying sludge from the separate treatment of digester liquids was studied. It is concluded that:

1. Sludge from the separate treatment of ammonium-rich liquids (e.g. from sludge digesting) harbors a different community composition of both ammonia- and nitrite oxidizing bacteria than activated sludge from municipal wastewater treatment plants (WWTPs). The former exhibits high substrate concentrations that favor r-strategists (*N. europaea*, *Nitrobacter*) with high saturation constants for ammonia and nitrite. K-strategists (*N. oligotropha*, *Nitrospira*) are dominating in municipal WWTPs due to their very low saturation constants.
2. The added nitrifiers are not able to adapt to the conditions prevailing in the WWTPs. At low substrate levels, they are outcompeted by the K-strategists and washed out depending on the sludge age and their decay rates. Loss of added bacteria in the effluent due to poor sedimentation in the secondary clarifier is negligible since the CAS and the MBRs showed the same behavior.
3. The enriched community composition is crucial for success and failure of bioaugmentation. The use of enriched nitrifying sludge from the separate treatment of digester liquids is not necessarily suited for the long-term enhancement of the nitrification performance. On the one hand, elevated nitrite concentrations in the effluent may occur if the seeded plant does not nitrify. On the other hand, its potential is limited due to the poor adaptation of the nitrifying bacteria if the seeded plant does already partially nitrify. Therefore, heightened diurnal variation of the influent N-K_j load due to the separate treatment of digester liquids increasing the required aerobic SRT cannot be compensated by adding enriched sludge into the biological tanks of a WWTP. Process engineering solutions which directly enrich the indigenous population in the return sludge stream (Salem *et al.*, 2003) avoid these drawbacks.

The recently developed method to quantify nitrifying bacteria (Manser *et al.*, 2005c) in combination with batch experiments should be further applied for tracking population dynamics in municipal WWTPs. As an example, it would allow to investigate the influence of the introduction of control strategies involving different environmental conditions regarding substrate and oxygen availability on the nitrifying bacteria.

Appendix

Table 3 Kinetic parameters of autotrophic bacteria at 20°C. Bold values are used for simulations.

Symbol	Characterization	Value	Units	Reference
μ_{AOBr}	max. growth rate of X_{AOBr}	0.6- 1.1 -1.6	d^{-1}	Prosser, 1989
μ_{AOBk}	max. growth rate of X_{AOBk}	0.8	d^{-1}	Bollmann <i>et al.</i> , 2002
μ_{NOBr}	max. growth rate of X_{NOBr}	0.6- 1.0 -1.4	d^{-1}	Prosser, 1989
μ_{NOBk}	max. growth rate of X_{NOBk}	0.75	d^{-1}	own measurements (unpublished)
$b_{\text{AOBr},\text{aer}}$	aerobic endogenous respiration rate of X_{AOBr}	0.22	d^{-1}	this study
$b_{\text{AOBk},\text{aer}}$	aerobic endogenous respiration rate of X_{AOBk}	0.15	d^{-1}	Manser <i>et al.</i> , 2005b
$b_{\text{NOBr},\text{aer}}$	aerobic endogenous respiration rate of X_{NOBr}	0.31	d^{-1}	this study
$b_{\text{NOBk},\text{aer}}$	aerobic endogenous respiration rate of X_{NOBk}	0.2	d^{-1}	Manser <i>et al.</i> , 2005b
$b_{\text{AOBr},\text{anox}}$	anoxic endogenous respiration rate of X_{AOBr}	0.01	d^{-1}	Manser <i>et al.</i> , 2005b
$b_{\text{AOBk},\text{anox}}$	anoxic endogenous respiration rate of X_{AOBk}	0.01	d^{-1}	Manser <i>et al.</i> , 2005b
$b_{\text{NOBr},\text{anox}}$	anoxic endogenous respiration rate of X_{NOBr}	0.01	d^{-1}	Manser <i>et al.</i> , 2005b
$b_{\text{NOBk},\text{anox}}$	anoxic endogenous respiration rate of X_{NOBk}	0.01	d^{-1}	Manser <i>et al.</i> , 2005b
$K_{\text{NH}_4,\text{AOBr}}$	saturation constant for ammonium of X_{AOBr}	6-14	$\text{g}_\text{N} \text{m}^{-3}$	this study, Stehr <i>et al.</i> , 1995
$K_{\text{NH}_4,\text{AOBk}}$	saturation constant for ammonium of X_{AOBk}	0.15	$\text{g}_\text{N} \text{m}^{-3}$	Manser <i>et al.</i> , 2005a
$K_{\text{NO}_2,\text{NOBr}}$	saturation constant for nitrite of X_{NOBr}	1-4-9	$\text{g}_\text{N} \text{m}^{-3}$	this study, Prosser, 1989
$K_{\text{NO}_2,\text{NOBk}}$	saturation constant for nitrite of X_{NOBk}	0.25	$\text{g}_\text{N} \text{m}^{-3}$	Manser <i>et al.</i> , 2005a

X_{AOBr} : Ammonia-oxidizing bacteria (r-strategists) from SBR reactor treating ammonium-rich wastewater

X_{NOBr} : Nitrite-oxidizing bacteria (r-strategists) from SBR reactor treating ammonium-rich wastewater

X_{AOBk} : Ammonia-oxidizing bacteria (K-strategists) from CAS or MBR treating municipal wastewater

X_{NOBk} : Nitrite-oxidizing bacteria (K-strategists) from CAS or MBR treating municipal wastewater

CHAPTER 4

Consequences of Mass Transfer Effects on the Kinetics of Nitrifiers

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Consequences of mass transfer effects on the kinetics of nitrifiers

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Abstract

The influence of membrane separation and mass transfer effects on the kinetics of nitrifiers was evaluated by running a membrane bioreactor (MBR) and a conventional activated sludge (CAS) plant in parallel. Both pilot plants were operated at the same sludge age and treated the same domestic wastewater. The half-saturation constants for the substrate were low in both MBR and CAS and did not differ significantly between the two processes ($K_{\text{NH}_4} = 0.13 \pm 0.05 \text{ g}_\text{N} \text{ m}^{-3}$ and $0.14 \pm 0.10 \text{ g}_\text{N} \text{ m}^{-3}$ and $K_{\text{NO}_2} = 0.17 \pm 0.06 \text{ g}_\text{N} \text{ m}^{-3}$ and $0.28 \pm 0.20 \text{ g}_\text{N} \text{ m}^{-3}$ for the MBR and CAS respectively). However, the half-saturation constants for oxygen exhibited a major difference between the two processes for both the ammonia-oxidizing (AOB) and nitrite-oxidizing (NOB) bacteria. The experiments yielded $K_{\text{O,AOB}} = 0.18 \pm 0.04$ and $0.79 \pm 0.08 \text{ g}_{\text{O}_2} \text{ m}^{-3}$ as well as $K_{\text{O,NOB}} = 0.13 \pm 0.06$ and $0.47 \pm 0.04 \text{ g}_{\text{O}_2} \text{ m}^{-3}$ (substrate only NO₂) for the MBR and CAS respectively. The higher K_{O} values of the CAS were attributed to mass transfer effects within the large flocs prevailing in the conventional system. In contrast, the sludge from the MBR consisted of very small flocs for which the diffusion resistance can be neglected. On the basis of these results, the implementation of mass transfer effects in activated sludge models is discussed and consequences for the operation of MBRs are highlighted.

Keywords: Activated sludge; Floc size distribution; Kinetics; Membrane Bioreactor; Modeling; Nitrification

1. Introduction

Membrane bioreactors (MBR) are increasingly considered for the treatment of domestic wastewater when new wastewater treatment plants are planned or existing ones redeveloped. Due to their low footprint and solid-free effluent, MBRs are particularly suitable if space is limited or strict requirements on effluent quality have to be met. The first full-scale plants have been put into operation in recent years by adapting the experience gained from conventional activated sludge plants (CAS).

However, the correct application of existing models from the conventional activated sludge process for MBR systems requires additional information about the kinetics of activated sludge in MBRs. Most investigations conducted so far on the sludge properties were carried out with respect to the membrane performance (e.g. Judd, 2004; Shimizu et al., 1997) and do not provide kinetic data. Manser *et al.* (2004) did not find a significantly different community composition of nitrifiers when comparing an MBR with a CAS. As a consequence, community-associated kinetic parameters, such as the yield coefficient or maximum growth rate, are expected to be equal in both processes. On the other hand, the half-saturation constants for oxygen and substrate used for modeling are typically lumped parameters that are additionally

influenced by potential mass transfer effects within the activated sludge flocs. Various authors found small floc sizes in MBRs compared with CAS systems (e.g. Cicek et al., 1999; Zhang et al., 1997), which would also support the supply of the complete MBR flocs with oxygen at low concentrations.

This study investigates the impact of membrane separation and mass transfer effects on the kinetics of nitrifiers. An MBR and a CAS pilot plant with the same settings were operated in parallel (Manser et al., 2004). Half-saturation constants were obtained by batch experiments. Measurement of the particle size distribution provided data on the sludge structure. The diffusion resistance is taken into account and its implication for modeling is discussed.

2. Materials and Methods

2.1 Pilot plants

A CAS and an MBR pilot plant (Manser et al., 2004) were operated in parallel to treat domestic wastewater following a typical diurnal hydraulic variation. The CAS and MBR treated wastewater corresponding to 60 ($18 \text{ m}^3\text{d}^{-1}$) and 2 ($0.56 \text{ m}^3\text{d}^{-1}$) population equivalents respectively. The oxygen concentration in the aerobic tanks was controlled between 2.5 and 3 g m^{-3} . Because a coarse bubble aeration is needed to induce a cross-flow at the membrane surface, the oxygen concentration was temporarily higher in the MBR. Both plants were operated with pre-denitrification and the sludge retention time (SRT) was kept at 20 days.

2.2 Batch experiments

A) Half-saturation constants for substrate (ammonia, nitrite). Activated sludge was aerated in batch reactors with a volume of 7.5 liters for at least two hours prior to adding the substrate to ensure that all biodegradable matter was consumed. The temperature and pH value were controlled and set to $15.0 \pm 0.2^\circ\text{C}$ and 7.50 ± 0.05 using HCl and NaOH in all experiments respectively. The oxygen concentration was controlled between 3 and 4 g m^{-3} in order to prevent oxygen limitation. AOB were inhibited by 10 g m^{-3} allylthiourea (K_S for nitrite only). Initial concentrations of the added substrate were between 5 and $8 \text{ g}_\text{N} \text{ m}^{-3}$. Samples were taken regularly until the substrate was entirely consumed. The substrate concentrations of the samples were measured according to *Standard Methods* (American Public Health Association, 1992). Three experiments were carried out for the calculation of each value.

B) Half-saturation constants for oxygen. Activated sludge was aerated in batch reactors with a volume of 1 liter for at least two hours prior to adding the substrate to ensure that all biodegradable matter was consumed. The temperature and pH value were controlled and set to $20.0 \pm 0.1^\circ\text{C}$ and 7.50 ± 0.01 in all experiments respectively. Nitritation and nitrification as well as heterotrophic endogenous respiration rates at various oxygen concentrations were measured by titration with NaOH and H_2O_2 respectively, as previously described (Ficara et al., 2000). AOB were inhibited by allylthiourea (10 g m^{-3}). Neither NOB nor heterotrophic bacte-

4. Consequences of mass transfer effects on the kinetics of nitrifiers

ria were chemically inhibited in any experiment. Prior to the measurement of the nitrification rates, the activated sludge was fed with wastewater containing a greater nitrite concentration for two days in order to increase the difference between endogenous respiration and the overall oxygen utilization rate (OUR). Four experiments were carried out for the calculation of each value.

2.3 Floc size distribution

The floc size distribution was measured regularly over a period of three months using a Mastersizer X (Malvern Instruments Ltd., Worcestershire, United Kingdom). The measurement was based on the principle of laser ensemble light scattering. However, activated sludge flocs are not inherently single, dense particles. Therefore, the floc number and sizes were determined once manually in order to obtain a comparison measurement. The microscopy was performed with an Olympus IX50 inverse microscope using 40x magnification. 100 µl of diluted activated sludge from the CAS were analyzed.

2.4 Floc density

Samples (80 ml) of activated sludge from the CAS and MBR were centrifuged for five minutes at 1000 rpm. The pellet was then weighed and the density of the floc calculated according to:

$$\rho_{\text{floc}} = \text{MLSS} \cdot \frac{80\text{ml}}{M_{\text{pellet}} \cdot \rho_{\text{pellet}}}$$

with: MLSS = Mixed liquor suspended solids ($\text{kg}_{\text{TSS}} \text{m}^{-3}$)

M_{pellet} = Pellet weight (g)

ρ_{pellet} = Pellet density ($\approx 1 \text{ g ml}^{-1}$)

2.5 Diffusion model

The diffusion model proposed by Beccari *et al.* (1992), that assumed spherical shape of the flocs, was slightly modified. The thickness of the external boundary layer was estimated by the Sherwood number correlation presented by Nicolella *et al.* (1998). The heterotrophic bacteria were distributed homogeneously within the whole floc, whereas the nitrifiers were concentrated in the inner part due to their low growth rates (Manser *et al.*, 2004). The floc size distribution was subdivided into 12 classes. The oxygen and substrate uptake rates of the bacteria were expressed as Monod terms by applying half-saturation constants measured in the MBR sludge. These are intrinsic values because the diffusion resistance can be neglected due to the small flocs. In the following, intrinsic half-saturation constants of the bacteria are indicated as K , apparent half-saturation constants including mass transfer effects as K' . The diffusion coefficients applied in water at 20 °C were 2.2, 1.7 and $1.7 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ for oxygen, ammonia and nitrite respectively (Milazzo, 1963; Schwarzenbach *et al.*, 2003).

The model was solved numerically using MATLAB (v7.0, The Math Works Inc, Natick, Ma, USA) equipped with a non-stiff ordinary differential equation solver (ODE45).

3. Results and discussion

3.1 Floc size distribution

Large differences between the CAS and the MBR were found. The flocs from the MBR were about ten times smaller than those from the CAS and confirm the results of other authors (Cicek *et al.*, 1999; Huang *et al.*, 2001; Zhang *et al.*, 1997). Since there is no selection for settleable flocs in MBR systems, the bacteria have no physical inducement to build up large flocs. The higher shear stresses in MBR systems due to the coarse aeration possibly also inhibit the growth of large flocs. The temporal variability of the floc size distribution (Table 1) is probably caused by the temperature decrease during the observed period (fall). While a relative comparison of the floc size distribution between the MBR and CAS sludge can reasonably be made by using the same measurement technique, absolute values have to be checked more critically. A comparison of the automatic laser scattering method with manual determination of floc number and size of a grab sample from the CAS revealed good agreement of the median values (Table 1). However, the distribution obtained manually was much narrower.

Table 1 Volumetric quantiles of the floc size distribution of activated sludge from the CAS and the MBR. *Upper part*: Mean values and standard deviations (temporal variability only) over a period of three months (n = 14). *Lower part*: Comparison of the automatic laser scattering method with manual determination of floc number and size for one sample from the CAS. A total of 230 flocs were enumerated and their mean diameter estimated.

	10%-quantile (µm)	50%-quantile (µm)	90%-quantile (µm)
CAS	73 ± 21	307 ± 72	733 ± 115
MBR	12 ± 3	35 ± 9	90 ± 23
Laser scattering CAS	112	408	1038
Manual counting CAS	262	419	629

As a consequence of the volumetric display, the distribution of the density functions is log-normal (Figure 1).

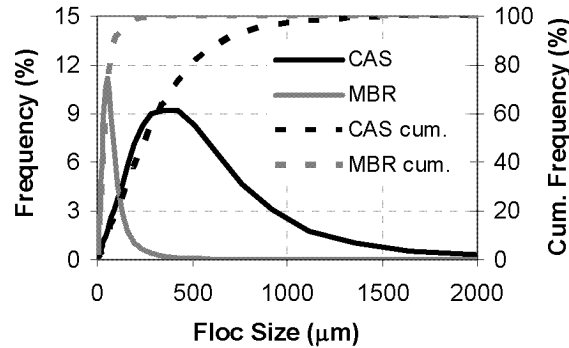


Figure 1 Volumetric floc size distributions of grab samples (within the investigated period of three months) from CAS and MBR measured with laser ensemble light scattering.

3.2 Floc density

The floc density expressed as $g_{TSS} m_{floc}^{-3}$ is an important parameter with regard to mass transfer effects within the flocs because it is directly proportional to the volumetric activity. Unfortunately, its determination turns out to be difficult, because no direct measurement technique is available. Bishop et al. (1995) found densities of biofilms from 30 up to $120 g_{TSS} m^{-3}$ depending on the thickness of the biofilm and the location within the biofilm. Maximum densities were found for biofilms with a thickness of 130-240 μm corresponding to the CAS floc diameters measured in this study (Table 1). Liss et al. (2001) examined the influence of the solid retention time on the floc structure and concluded that sludge flocs at higher SRTs (16 and 20 days) were more compact and physically more stable than those at lower SRTs (4 and 9 days). Rather dense flocs should therefore be expected from the examined pilot plants (SRT = 20 days). However, the densities determined in this study were in the lower range of these findings. After centrifugation of the activated sludges, the pellet comprised 10-14 Vol% and 14-22 Vol% of the total suspension for the CAS and MBR sludge respectively. This corresponds to similar floc densities of 25-35 and 27-42 $g_{TSS} m_{floc}^{-3}$ for the CAS and MBR sludge respectively. Using the data from manual counting of the flocs, a floc density of only 18 $g_{TSS} m_{floc}^{-3}$ was calculated for the CAS sludge. The Sludge Volume Index (SVI) of the CAS sludge was about $50 ml g_{TSS}^{-1}$, corresponding to a floc density of 20 $g_{TSS} m_{floc}^{-3}$.

In other words, the applied techniques only allow a rough estimation of the floc density and the data from the literature vary considerably. The sensitivity of this parameter is consequently discussed in Chapter 3.3.

3.3 Kinetics

Different bacteria populations have inherently distinct ecophysical properties. The analysis of the community composition revealed the same populations of nitrifying bacteria in both MBR and CAS (Manser et al., 2004). Consequently, equal kinetic properties can be expected. However, mass transfer effects additionally influence the kinetics of the nitrifiers in a different way (Figure 2). Oxygen and ammonia concentrations decrease across the floc, whereas nitrite

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is mainly produced within the floc by the AOB and immediately consumed by the NOB. As a consequence, the availability of nitrite is unaffected by diffusion. In addition, the outer purely heterotrophic layer already reduces the oxygen available for nitrification.

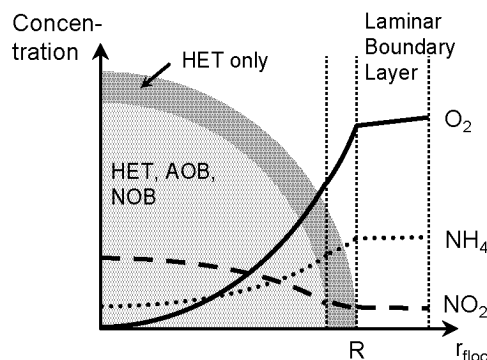


Figure 2 Schematic 2D slice of a spherical activated sludge floc with radius R and a purely heterotrophic outer layer

A diffusion model allows the effect of the mass transfer on the oxygen and substrate utilization rates to be described. However, several uncertainties affect the model output. They stem firstly from the uncertainty of the model structure, e.g. the assumptions of spherical shape and uniform density of the flocs. But uncertainties are also introduced by the limited knowledge of the parameters. Since these two sources of uncertainty are not independent of each other, the uncertainty of the parameters can partly reflect that of the model structure. As an example, the uncertainty of the parameter of *floc density* is responsible not only for the limited knowledge of the actual floc density but also for the variability of the floc density within and between the flocs.

A local sensitivity analysis revealed that the parameter with the highest sensitivity to the model output is the floc size. An increase of 100 μm in the mean floc diameter results in a $K_{O'}$ value that is about 20-40% higher (Figure 3D, G). Furthermore, the floc diameter is a rather theoretical value, since the assumption of a spherical shape of the flocs is in reality not true. Therefore, the uncertainty in the absolute floc size contributes significantly to the uncertainty of the calculated $K_{O'}$ values. Apart from the floc size, the floc density and the reduction of the diffusion coefficients within the floc ($D_{\text{floc}}/D_{\text{water}}$) were most sensitive to the model output (Figure 3). Unfortunately, these parameters cannot be readily measured and the data from the literature vary considerably. The diffusion coefficients within the floc were found to be between 30% and 60% of those in pure water (Bishop et al., 1995; Matson and Characklis, 1976). Bishop *et al.* (1995) found floc densities from 30 up to 120 $\text{kg}_{\text{TSS}} \text{m}_{\text{floc}}^{-3}$ in biofilms. However, both the diffusion coefficient and the floc density were strongly dependent on the spatial position within the biofilm. Assuming the outer purely heterotrophic layer to be 20% of the floc radius, the modeled $K_{O, \text{AOB'}}$ increased by about 20% compared to a floc without a heterotrophic layer (Figure 3F). Although the diffusion coefficient of ammonia is even slightly smaller than that of oxygen, the utilization rate of ammonia is over three times lower than that of oxygen, leading to only small mass transfer effects due to the stoichiometry

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(Figure 3A-C). In addition, heterotrophic activity in the form of ammonia turnover can be neglected.

The results clearly reveal that the mass transfer limitation due to diffusion plays a significant role in activated sludge flocs, but is negligible for flocs with a diameter smaller than 100 μm (Figure 3A, D, G). Consequently, the K' values of MBR sludge reflect the intrinsic K values of the bacteria, because more than 90% of the floc volume in the sludge consisted of flocs smaller than 100 μm (Table 1).

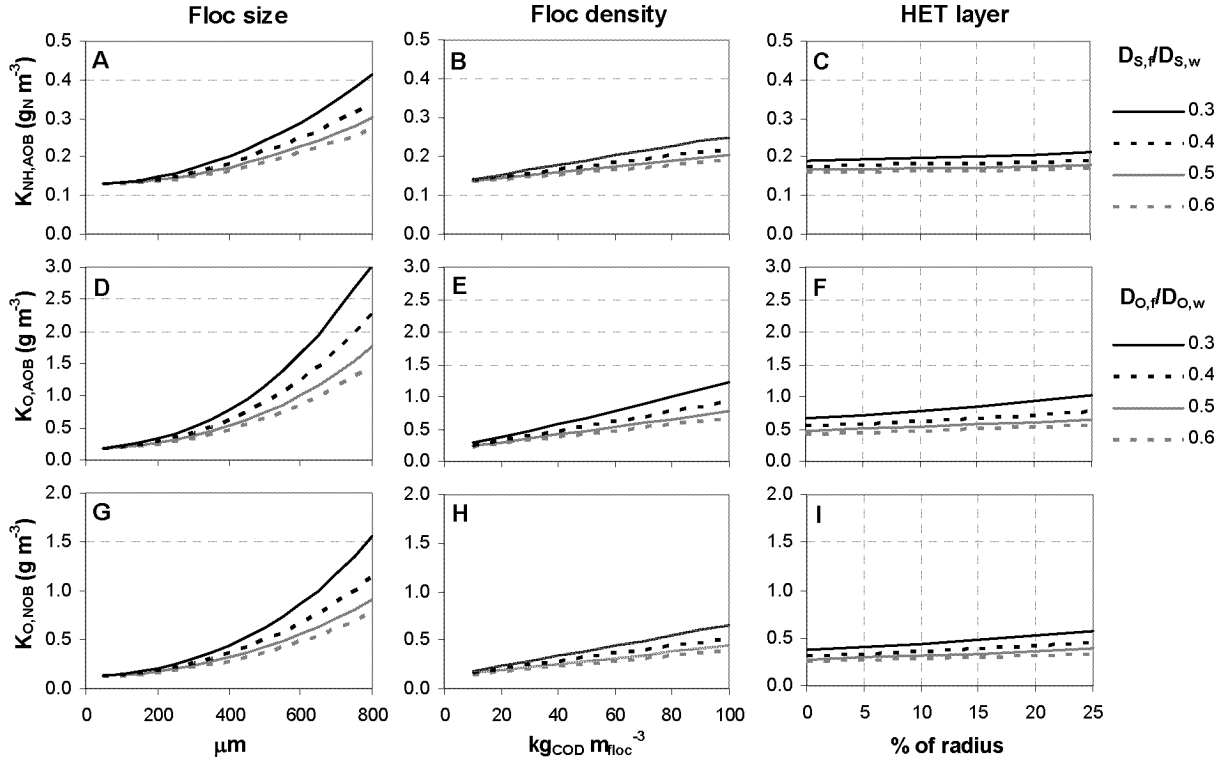


Figure 3 Calculated half-saturation constants for substrates (A-C) and oxygen (D-I) as a function of the reduction of the diffusion coefficient within the floc (D_f/D_w) and floc size (A, D, G), floc density (B, E, H) and thickness of the heterotrophic layer (C, F, I). Default parameter values used were: floc density = 60 $\text{kg COD m}_{\text{floc}}^{-3}$, thickness of the heterotrophic layer = 10% of floc radius and floc size = 400 μm . Calculation of rates for K_{NH_4} : $r_{AOB} = 35 \text{ g}_{NH_4-N} \text{ kg COD}^{-1} \text{ d}^{-1}$. Calculation of rates for $K_{O, AOB}$: $r_{AOB} = 130 \text{ g}_{O_2} \text{ kg COD}^{-1} \text{ d}^{-1}$, $r_{NOB} = 35 \text{ g}_{O_2} \text{ kg COD}^{-1} \text{ d}^{-1}$, $r_{HET} = 100 \text{ g}_{O_2} \text{ kg COD}^{-1} \text{ d}^{-1}$ (= endogenous respiration). Calculation of rates for $K_{O, NOB}$ (NOB slightly enriched): $r_{NOB} = 45 \text{ g}_{O_2} \text{ kg COD}^{-1} \text{ d}^{-1}$, $r_{HET} = 90 \text{ g}_{O_2} \text{ kg COD}^{-1} \text{ d}^{-1}$ (= endogenous respiration).

Half-saturation constants for the substrate (K_s)

Measurement of the degradation of the substrates (Figure 4) and subsequent estimation of the half-saturation constants yielded low values for both AOB and NOB (Table 2). Furthermore, no significant difference was found between the CAS and the MBR. Mass transfer plays only a minor role here because the conversion rate of ammonia is lower than that of oxygen and heterotrophic activity with respect to ammonia and nitrite can be neglected (Figure 3A-C). However, the K_{NOB}' values of the CAS varied within a broad range over time. This variation

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may be explained by uncertainties associated with the estimation of such low values. But higher K_{NOB} values also correlate with higher floc sizes (Table 2) and therefore imply the influence of diffusion resistance within large flocs (Figure 3A).

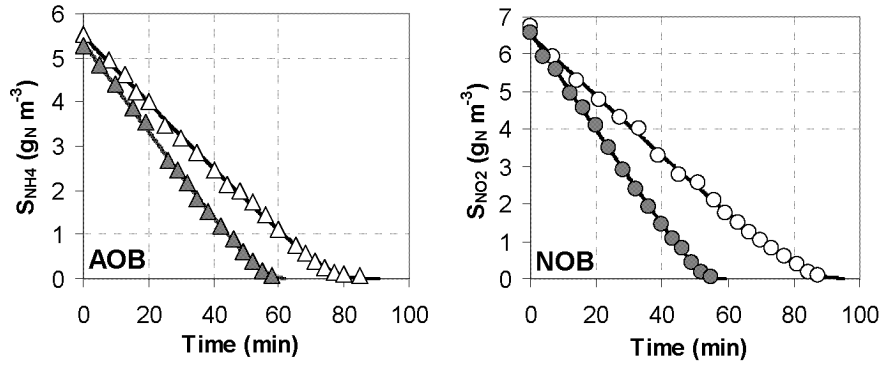


Figure 4 Examples of measured concentration profiles used for the estimation of $K_{NH, AOB}$ and $K_{NO, NOB}$. Lines represent the best fit (chi2) to the Monod model.

It should be emphasized that nitrite is normally produced within the flocs where mass transfer effects can be neglected. However, the experimental setup did not exclude potential diffusion resistance. The equal K_S values of both CAS and MBR can largely be explained by the same prevailing composition of nitrifier communities in both systems (Manser et al., 2004), which probably exhibited similar ecophysical properties. More surprising were the low values of both AOB and NOB, since mostly higher values were reported in previous studies (Bollmann *et al.*, 2002; Prosser, 1989; Schramm *et al.*, 1999). The analysis of the community composition revealed that the AOB were dominated by members of the *Nitrosomonas oligotropha* lineage, whereas NOB belonged to the genus *Nitrospira* (Manser et al., 2004). Both groups are regarded as typical K -strategists with high substrate affinities (Gieseke et al., 2001; Schramm et al., 1999). Stehr *et al.* (1995) found $K_{NH, AOB}$ values of 0.42-1.05 $g_N m^{-3}$ for members of the *Nitrosomonas oligotropha* lineage, which slightly exceed the values estimated in this study (Table 2). We assume that either the AOB in the pilot plants were able to adapt to even lower ammonia concentrations or other AOB were present which are beyond the resolution of the detection method used (FISH). Schramm *et al.* (1999) obtained a $K_{NO, NOB}$ value of 0.14 $g_N m^{-3}$ for members of the genus *Nitrospira*, which is confirmed by the values found in this study (Table 2). Finally, the dynamic measurement of the ammonium concentrations does not reflect the steady-state conditions of continuously fed systems and may have led to very low values.

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Table 2 Estimated intrinsic K_S values for MBR and apparent K_S' for CAS of AOB and NOB at 15°C. Standard errors from parameter estimation are given. 50%-quantiles of the measured floc size distributions are listed. The experiments were distributed over a period of six months.

	$K_{NH, AOB}$ ($g_N m^{-3}$)	Floc size 50%-q. (μm)	Rate AOB ($g_N kg_{COD} d^{-1}$)	$K_{NO, NOB}$ ($g_N m^{-3}$)	Floc size 50%-q. (μm)	Rate NOB ($g_N kg_{COD} d^{-1}$)
MBR	0.10 ± 0.06	73	31 ± 1.5	0.12 ± 0.02	73	28 ± 0.9
	0.15 ± 0.03	79	32 ± 0.9	0.22 ± 0.04	73	35 ± 1.2
	0.12 ± 0.04	78	38 ± 1.1	0.15 ± 0.02	78	29 ± 0.8
	$K_{NH, AOB}'$			$K_{NO, NOB}'$		
CAS	0.07 ± 0.02	315	41 ± 1.0	0.50 ± 0.03	593	46 ± 0.9
	0.20 ± 0.06	529	42 ± 1.5	0.22 ± 0.04	406	27 ± 0.8
	0.16 ± 0.03	418	39 ± 0.8	0.11 ± 0.02	184	31 ± 1.0

Half-saturation constants for oxygen (K_O)

Table 3 summarizes the half-saturation constants obtained for oxygen with AOB, NOB and heterotrophic bacteria of the MBR. Since mass transfer effects can be neglected, the simple Monod model was used for the estimation of the parameters. Interestingly, the K_O ($= K_O'$) values for AOB and NOB were found not to be significantly different, which contrasts with previous findings stating higher K_O values for NOB than AOB (Belser, 1979; Prosser, 1989). However, kinetic data from previous studies are based on experiments with NOB belonging to the genus *Nitrobacter*, which is regarded as a typical *r*-strategist with low affinity to oxygen and the substrate. Conversely, NOB belonging to the genus *Nitrospira* are also thought to possess a higher affinity to oxygen (Schramm et al., 1999). The K_O values of the MBR correspond to values found in the literature for pure cultures (Belser, 1979; Gieseke *et al.*, 2001; Prosser, 1989). It should be considered that neither nitrification nor heterotrophic endogenous respiration were chemically inhibited during nitrification and therefore simultaneously consumed oxygen. Since heterotrophic endogenous respiration influences the measurement of the nitrification rate (consumption of H_2O_2), the half-saturation constant of heterotrophic bacteria during endogenous respiration was determined. Its estimation produced a very low value, which was consistent with previous findings (Belser, 1979; Wiesmann, 1994).

Table 3 Estimated intrinsic K_O values ($=$ apparent K_O' values, no diffusion resistance) for AOB, NOB and heterotrophic bacteria (endogenous respiration) of MBR sludge. Mean values and standard deviations (four experiments each) at 20 °C.

$K_{O, AOB}$ ($g_{O_2} m^{-3}$)	$K_{O, NOB}$ ($g_{O_2} m^{-3}$)	$K_{O, HET, endo}$ ($g_{O_2} m^{-3}$)
0.18 ± 0.04	0.13 ± 0.06	0.05 ± 0.02

In contrast to the MBR, the estimation of the K_O' values for larger flocs must take mass transfer effects into account (Figure 3D, G). The application of the diffusion model to measured data of the CAS showed that the observed values could well be explained by diffusion resistance (Figure 5). However, a high floc density ($80 kg_{COD} m_{floc}^{-3}$) and low $D_{O,f}/D_{O,w}$ ratio (0.3)

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had to be assumed in order to explain the differences between the CAS and the MBR purely by mass transfer effects within the floc. Although the flocs from the CAS were very compact (low SVI) and the applied parameter values are still in the range of the published data (Bishop et al., 1995; Matson and Characklis, 1976), other factors such as larger floc size, different ecophysical properties of the nitrifiers between the CAS and MBR systems or mass transfer effects within the dense aggregates of nitrifiers may have contributed to the measured dissimilarities. In particular, the non-homogenous distribution of AOB and NOB in the flocs may lead to dissolved oxygen (DO) gradients around the aggregates. In addition, Manser *et al.* (2004) found larger aggregates in sludge from CAS than from MBR. The parallel measurement of the overall oxygen uptake rate (as the H_2O_2 consumption rate) and the nitrification rate (as the NaOH consumption rate) confirmed that the AOB were more inhibited at low oxygen concentrations than heterotrophic bacteria (Figure 5C). Apart from the higher $K_{O, \text{AOB}}$ value compared to $K_{O, \text{HET}}$ (Table 3), the outer purely heterotrophic layer probably enhanced this effect.

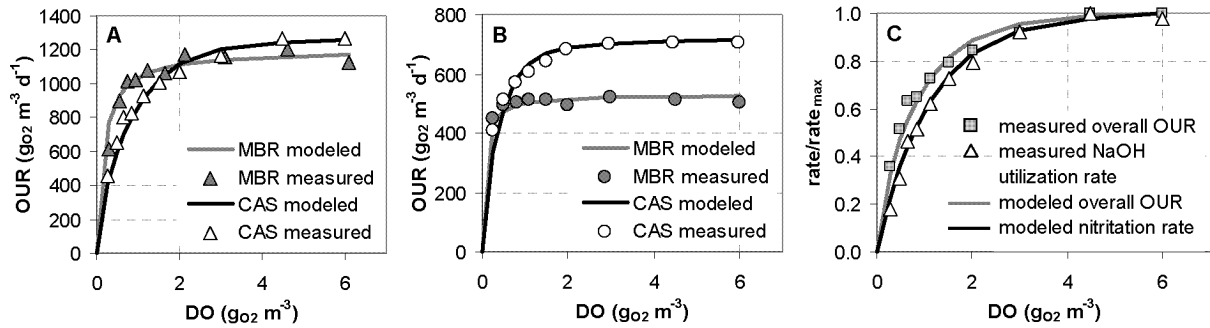


Figure 5 Examples of measured and calculated oxygen utilization rates (OUR) as a function of the bulk oxygen concentration for the CAS with (B) and without (A) inhibition of AOB. Substrate concentrations were non-limiting. Estimated parameters: floc density = $80 \text{ kg}_{\text{COD}} \text{ m}_{\text{floc}}^{-3}$, thickness of the heterotrophic layer = 10% of floc radius, $D_{O,f}/D_{O,w} = 0.3$. Comparison of nitrification rate (measured as NaOH utilization rate) with overall OUR (measured as H_2O_2 utilization rate) of CAS (C). For the rates used for the calculations, see the legend of Fig. 3.

Accordingly, the estimation of the apparent half-saturation constants for the CAS from the experimental data led to significantly higher values compared to the MBR (Table 4). It should be considered that the temporal variability of the conventional activated sludge properties strongly influences the mass transfer effects and therefore the actual K_O' values. A lower $K_{O, \text{AOB}}'$ value ($0.50 \text{ gO}_2 \text{ m}^{-3}$) was found with conventional activated sludge from a second period, which was associated with smaller flocs (50%-quantile: $197 \mu\text{m}$) and a high SVI value ($>250 \text{ ml g}_{\text{TSS}}^{-1}$). In contrast, the sludge properties of the MBR did not vary significantly (data not shown).

4. Consequences of mass transfer effects on the kinetics of nitrifiers

Table 4 Estimated apparent K_O' values for AOB, NOB and heterotrophic bacteria (endogenous respiration) of CAS sludge at 20 °C. Standard errors of parameter estimation are given. Model parameters used: floc density = 80 kg_{COD} m_{floc}⁻³, thickness of the heterotrophic layer = 10% of floc radius, $D_{O,f}/D_{O,w} = 0.3$.

$K_{O,AOB}'$ (gO ₂ m ⁻³)	$K_{O,NOB}'$ (gO ₂ m ⁻³)	$K_{O,HET}'$ (gO ₂ m ⁻³)
0.79 ± 0.08	0.47 ± 0.04 ¹⁾	0.16 ± 0.02

¹⁾ Without AOB activity

The diffusion model allows the K_O' values to be calculated as a function of the active processes (Figure 6). The microbial activity of NOB (Figure 6A), AOB (Figure 6B) and heterotrophic bacteria (Figure 6C) influences the oxygen consumption within the floc and therefore also alters the diffusion resistance. While the activity of NOB only slightly raises the $K_{O,AOB}'$, the maximum activity of AOB leads to a 30% increase of the $K_{O,NOB}'$. Nonetheless, this reduction of NOB activity due to high AOB activity cannot yet explain the occurrence of elevated nitrite concentration in the effluent of CAS systems (assuming homogenous spatial distribution of nitrifiers). $K_{O,NOB}'$ is also lower than $K_{O,AOB}'$ during high AOB activity due to the slightly lower intrinsic K_O value of the NOB used for the calculations (Table 3). Conversely, heterotrophic activity could noticeably inhibit nitrification by strongly increasing the K_O' values of the nitrifiers. The presence of an outer heterotrophic layer would obviously enhance this effect (Figure 6C). Although diffusion resistance increases with decreasing temperature, K_O' values become smaller with diminishing temperature due to substantially reduced microbial activity (Figure 6D).

Consequently, DO control for CAS could be optimized on the basis of these findings. It would certainly be possible to drop the DO to low levels during low nitrogen loading periods, whereas the DO should be increased to higher levels to counteract the diffusion limitation under high nitrogen loading situations. This would save aeration costs, limit the aerobic consumption of COD (thus leaving more available for denitrification) and improve the possibility of achieving simultaneous nitrification and denitrification.

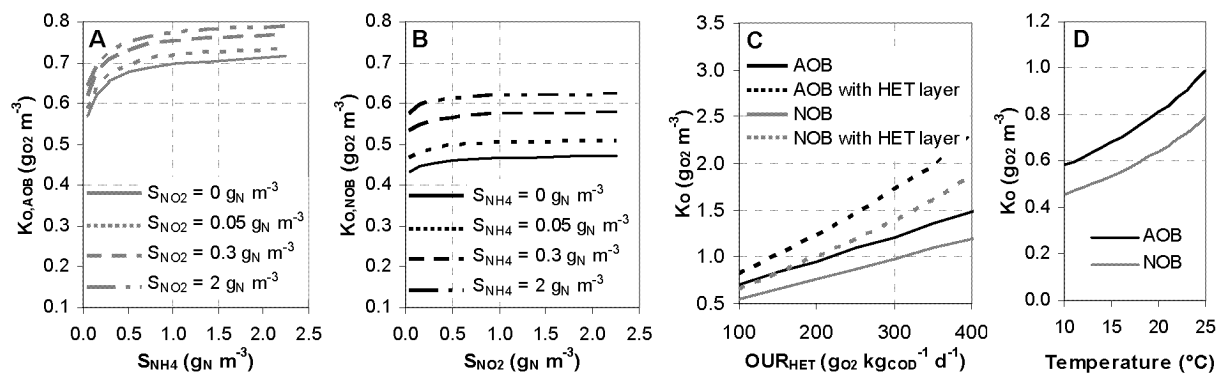


Figure 6 Calculated half-saturation constants for oxygen of the CAS depending on the active processes at 20°C (except D). Endogenous respiration = 100 gO₂ kg_{COD} d⁻¹, HET layer of 10% of radius is assumed. $K_{NH4} = 0.15$ g_N m⁻³, $K_{NO2} = 0.15$ g_N m⁻³ (Table 2). Substrate levels indicate steady state concentrations, e.g. $S_{NO2} = 0$ g_N m⁻³ means that NOB are not active.

3.4 Implications for modeling of nitrification

The reported results have consequences for modeling nitrification. Mass transfer effects play an important role in CAS but not in MBR systems. Since computation times still appear to be prohibitive for extending activated sludge models by a diffusion model, the diffusion resistance can reasonably be reflected in dynamic half-saturation constants. Accordingly, the K_O' values in CAS systems depend on the active processes and the temperature (Figure 6). On the basis of these results, the following line-fit formulas for the calculation of the K_O' values were derived:

$$K_{O, AOB, 20^\circ C} = K_{O, AOB, 20^\circ C, \min} + a_{HET} \cdot \frac{S_S}{K_S + S_S} + a_{AOB, 1} \cdot \frac{S_{NH_4}}{K_{NH_4, AOB} + S_{NH_4}} + a_{NOB} \cdot \frac{S_{NO_2}}{K_{NO_2, NOB} + S_{NO_2}}$$

$$K_{O, NOB, 20^\circ C} = K_{O, NOB, 20^\circ C, \min} + a_{HET} \cdot \frac{S_S}{K_S + S_S} + a_{AOB, 2} \cdot \frac{S_{NH_4}}{K_{NH_4, AOB} + S_{NH_4}}$$

$$K_{O, \dots, T} = K_{O, \dots, 20^\circ C} \cdot e^{0.036(20-T)}$$

The K_O' values are divided into a constant and a process-dependent fraction. The constant fractions can be estimated from the results shown in Figure 6 ($K_{O, AOB, 20^\circ C, \min} = 0.60 \text{ gO}_2 \text{ m}^{-3}$, $K_{O, NOB, 20^\circ C, \min} = 0.47 \text{ gO}_2 \text{ m}^{-3}$, both including heterotrophic endogenous respiration of $100 \text{ gO}_2 \text{ kgCOD}^{-1} \text{ d}^{-1}$). Similarly, the contributions of nitrification ($a_{AOB, 1} \approx 0.1 \text{ gO}_2 \text{ m}^{-3}$, $a_{AOB, 2} \approx 0.2 \text{ gO}_2 \text{ m}^{-3}$, Figure 6A-B) and nitrification ($a_{NOB} \approx 0.1 \text{ gO}_2 \text{ m}^{-3}$, Figure 6A) can also be found. The OUR due to heterotrophic growth was correlated with the availability of readily biodegradable substrate S_S ($a_{HET} \approx 0.8 \text{ gO}_2 \text{ m}^{-3}$, $K_S' \approx 2 \text{ gCOD m}^{-3}$, Figure 6C). The influence of the nitrite concentration on the $K_{O, NOB}$ was neglected. Finally, the K_O' values must be adjusted to the temperature according to Figure 6D.

The formulas were incorporated in a common activated sludge model (ASM3, Gujer *et al.*, 1999) in order to test their sensitivity in the nitrification process. A common setup of a CAS and a MBR with two completely mixed reactors and one secondary settling tank (CAS only) was consequently chosen. The sludge age was set to 10 days. Both reactors were aerated with a set point of $S_O = 1 \text{ gO}_2 \text{ m}^{-3}$, since diffusion effects are negligible at high oxygen concentrations. All simulations were carried out with a typical domestic influent as shown in Figure 7. The results indicate that heterotrophic growth only marginally increases the autotrophic K_O' values for typical domestic COD dynamics and leads to a relatively small diurnal variation of the calculated K_O' values. As a consequence, mean K_O' values ($K=\text{const}$) yield similar effluent concentrations at $S_O = 1 \text{ gO}_2 \text{ m}^{-3}$ (Figure 7). In contrast, the application of constant K_O' values independently of the system may lead to large errors. As an example, the transfer of K_O' values from CAS systems to MBR systems results in a significant underestimation of the nitrification performance (Figure 7). The loss of performance during cold seasons owing to diminished microbial activity is partly lessened by the reduced diffusion resistance.

4. Consequences of mass transfer effects on the kinetics of nitrifiers

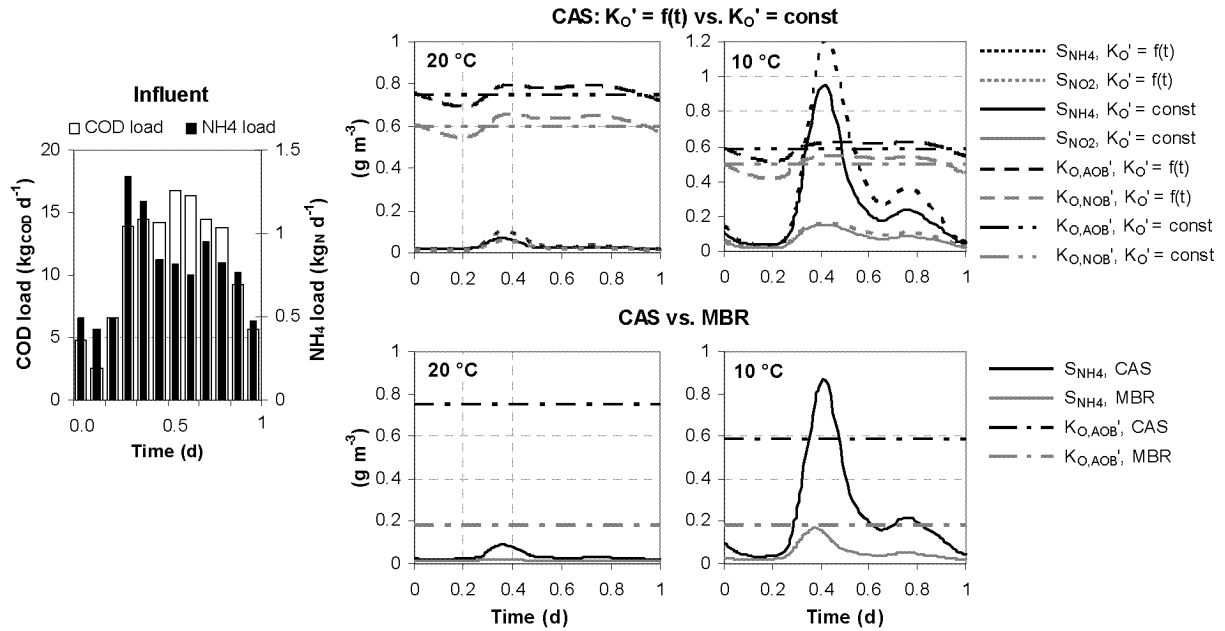


Figure 7 Influent load and calculated effluent concentrations of an aerobic CAS and MBR ($S_{O_2} = 1 \text{ g}_{O_2} \text{ m}^{-3}$) at 20 and 10 °C. $K_{O_2} = \text{const}$ corresponds to mean values, dependent on temperature only.

These considerations are based on the assumption that the floc structure remains constant under various conditions. However, floc sizes may vary significantly over time due to fluctuations of the influent composition or temperature. Moreover, control strategies (e.g. reduced aeration in order to enhance nitrogen elimination) may alter the floc structure associated with different mass transfer effects. Differences between WWTPs are likely to occur because of different influent compositions, loads or operation. Therefore, the proposed model should be validated in real systems on its improved ability to reproduce ammonia and nitrite dynamics.

4. Conclusions

In this paper, we have demonstrated the consequences of mass transfer effects on the kinetics of nitrification. A conventional activated sludge (CAS) and a membrane bioreactor (MBR) pilot plant were operated in parallel to treat the same domestic wastewater. Half-saturation constants for both substrate and oxygen were estimated from batch experiments.

It is concluded that diffusion resistance is negligible for MBR systems because the membrane separation leads to small floc sizes. Consequently, the measured (apparent) half-saturation constants in MBRs reflect the low intrinsic K values of the bacteria. MBR systems may therefore be operated at low oxygen concentrations ($1 \text{ g}_{O_2} \text{ m}^{-3}$), which would save aeration energy. Additionally, the internal recycle from the aerobic to the anoxic zone should not be directed from the membrane compartment (high DO due to necessary cross-flow aeration) in order to minimize the oxygen transfer from the aerobic to the anoxic zone. In contrast, mass transfer effects play a key role within the large flocs prevailing in the CAS. The apparent half-

4. Consequences of mass transfer effects on the kinetics of nitrifiers

saturation constants for oxygen are significantly higher in CAS because they include both the intrinsic K values of the bacteria and the effect of the diffusion resistance. Accordingly, simultaneous nitrification and denitrification can be achieved in CAS due to the DO gradients within the large flocs. However, the mass transfer effects depend strongly on variable parameters such as floc size and floc density, which may vary significantly between different WWTPs. Apparent K_{BOB}' values are thus system-dependent parameters and must be determined individually. The implementation of these findings in activated sludge models is a first step towards the understanding of ammonia and nitrite dynamics in CAS, particularly at low oxygen concentrations. Temperature-dependent static K_O' values seem to yield reasonable results for common CAS with typical diurnal variation, whereas a highly variable influent probably requires dynamic K_O' values depending on the active processes.

Accordingly, future research is needed in order to validate the proposed model extension. However, the impact of membrane separation on other kinetic parameters such as decay constants should also be examined in order to provide a more comprehensive parameter set for the modeling of MBR.

CHAPTER 5

Decay Processes of Nitrifying Bacteria

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Submitted to Water Research

Decay Processes of Nitrifying Bacteria

Reto Manser, Willi Gujer, Hansruedi Siegrist

Abstract

The extension of wastewater treatment plants from only COD to nutrient removal leads to increased sludge ages and exposes bacteria to different oxidation-reduction potential (ORP) conditions. Moreover, new technologies like membrane bioreactors are operated at higher activated sludge concentrations associated with elevated sludge ages. Consequently, profound knowledge about decay processes and kinetics becomes increasingly important. Therefore, batch experiments were carried out in order to study the effect of membrane separation and different ORP conditions on the decay of ammonia- and nitrite-oxidizing bacteria. It was found that decay at anoxic conditions is negligible for both ammonia- and nitrite-oxidizing bacteria. No significant differences between membrane and conventional activated sludge were detected. Likewise, aerobic decay of ammonia- and nitrite-oxidizing bacteria did not significantly diverge. However, the measured loss of autotrophic activity was only partly explained by the endogenous respiration concept as incorporated e.g. in the activated sludge model no. 3 (ASM3). Unlike nitrite-oxidizing bacteria, ammonia-oxidizing bacteria showed a pronounced decay of their enzymes. Based on an extended ASM3, the significance of these findings on wastewater treatment is discussed.

Keywords: Activated sludge; Decay; Enzyme kinetics; Membrane Bioreactor; Modeling; Nitrification

1. Introduction

Autotrophic decay rates have a significant impact on the nitrification performance and therefore on the design and analysis of wastewater treatment plants. Safety of the plant performance against wash-out or overload directly depend on these kinetic parameters. Furthermore, the extension of the biological treatment steps from only COD removal to plants with nitrification, denitrification and biological phosphorus removal exposes bacteria to different oxidation-reduction potential (ORP) conditions, which appear to influence the decay rates (Lee and Oleszkiewicz, 2003; Siegrist *et al.*, 1999). Additionally, membrane bioreactors are operated at higher activated sludge concentrations associated with elevated sludge ages.

State of the art models consider loss of biomass as a single process proportional to the biomass concentration (e.g. lysis, endogenous respiration). Looking into more details, loss of biological activity can be the result of mechanisms like: predation by protozoa, endogenous respiration or lysis due to adverse environmental conditions (Van Loosdrecht and Henze, 1999). In the maintenance process – as another concept – external substrate is used to keep the current biological activity, but the amount of bacteria is not altered. Predation and lysis both reduce cell number, biomass and activity, whereas endogenous respiration – from a microbiological point of view – only leads to a loss of activity and slightly reduces biomass. Finally,

enzyme degradation causes a loss of activity, which is rapidly restored if substrate becomes available.

However, it is not clear, whether these mechanisms are reasonably described with only one process. In addition, previous studies merely provide kinetic data on decay in activated sludge systems combining nitrification as one process (summarized in Martinage and Paul, 2000). Possible differences between ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) may contribute to a better comprehension of nitrite dynamics. Eventually, from many investigations it does not become clear whether simultaneous growth of nitrifying bacteria on decay products from heterotrophic bacteria or product inhibition due to accumulation of nitrate was taken into account.

The goal of this study was the investigation of decay processes for both AOB and NOB at different ORP conditions and for different systems (conventional and membrane bioreactor). Loss of activity was measured in batch experiments by respirometry. Underlying processes are discussed and consequences for modeling of activated sludge systems presented.

2. Materials and Methods

2.1 Activated sludge samples

Samples were taken from a conventional activated sludge system (CAS) and a membrane bioreactor (MBR) pilot plant (Manser *et al.*, 2004), which were operated in parallel treating domestic wastewater following a typical diurnal hydraulic variation. The CAS and the MBR treated the wastewater corresponding to 60 ($18 \text{ m}^3 \text{ d}^{-1}$) and 2 ($0.56 \text{ m}^3 \text{ d}^{-1}$) population equivalents, respectively. Oxygen concentration in the aerobic tanks was controlled between 2.5 and 3 g m^{-3} . Due to the coarse bubble aeration inducing a cross-flow at the membrane surface, the oxygen concentration was temporarily higher in the MBR. Both plants were operated with pre-denitrification and the sludge retention time (SRT) was kept at 20 days.

2.2 Batch experiments

Activated sludge samples were placed in a batch reactor that either was continuously aerated (decay under aerobic conditions), only stirred (decay at anoxic conditions) or a combination of both for a period of 7-10 days (Figure 1). For the anoxic experiment, nitrate (NaNO_3) was manually added in order to prevent anaerobic conditions. Furthermore, the reactor was aerated once a day for five minutes enabling the nitrifiers to restore their ATP pool. An experiment with alternating stirred (8h) and aerated (16 hours) phases simulating a typical wastewater treatment plant with 33% anoxic volume was conducted in order to investigate a possible effect of the feast-famine phenomenon on decay (Lee and Oleszkiewicz, 2003). At intervals of one day, one liter of activated sludge was withdrawn from the batch reactor and centrifuged at 3000 rpm for seven minutes. Subsequently, the pellet was resuspended with effluent of the MBR (permeate) and placed in a secondary batch reactor. The main purpose of the centrifuga-

tion step was to provide low nitrate (ammonia for anoxic experiment) concentrations for the measurements, because a nitrate concentration of only $50 \text{ g}_\text{N} \text{ m}^{-3}$ was found to lead to a 20% reduction of the nitrataion rate (data not shown). After determining the base oxygen utilization rate (OUR; Figure 1: A), samples were successively spiked with nitrite (NaNO_2 , $6\text{--}8 \text{ g}_\text{N} \text{ m}^{-3}$) and ammonia (NH_4Cl , $10\text{--}18 \text{ g}_\text{N} \text{ m}^{-3}$), leading to maximum nitrataion (Figure 1: B) and nitrification rates (Figure 1: C). All experiments were carried out at pH 7.5 and 20.0°C . pH was controlled using HCl and NaHCO_3 . Oxygen concentration was controlled between 3 and $4 \text{ g}_{\text{O}_2} \text{ m}^{-3}$.

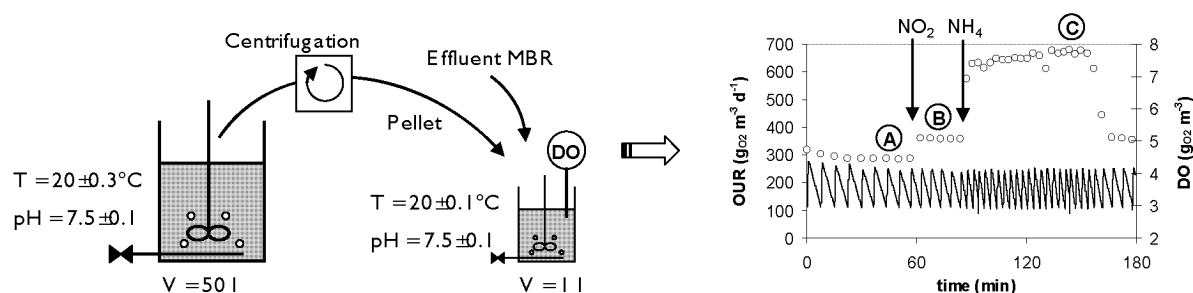


Figure 1 Experimental setup for batch experiments. A = base oxygen utilization rate (heterotrophic rate), B = max. nitrataion rate, C = max. nitrification rate.

2.3 Fluorescence In Situ Hybridization (FISH)

Samples were taken at maximum OUR of AOB and fixed in 4% paraformaldehyde as described by Amann *et al.* (1990). Ultrasonification (35 kHz, five minutes) was applied to fixed samples from the CAS prior to hybridization in order to break up large flocs. *In situ* hybridizations of cells were performed with fluorescently labeled, rRNA-targeted oligonucleotide probes according to Manz *et al.* (1992) (Table 1). Oligonucleotide probes were obtained from Microsynth (Balgach, Switzerland) and Thermo Hybaid (Interactiva Division, Ulm, Germany).

All samples hybridized with oligonucleotide probes were embedded in Citifluor (Citifluor, Canterbury, United Kingdom) prior to microscopic observation. Epifluorescence microscopy was done on an Olympus BX50 microscope, equipped with filters HQ-CY3 and HQ-FLUOS (both from Analysentechnik AG, Tübingen, Germany). Quantification of nitrifying bacteria was carried out according to Manser *et al.* (2005c).

Table 1 Oligonucleotide probes and hybridization conditions applied in this study.

Probe	Target organisms	Reference
NEU ^a	Most halophilic and halotolerant ammonia oxidizers in the beta-subclass of <i>Proteobacteria</i>	Wagner <i>et al.</i> , 1995
NmII	Many members of the <i>Nitrosomonas communis</i> lineage	PommereningRoser <i>et al.</i> , 1996
Nmo218	Many members of the <i>Nitrosomonas oligotropha</i> lineage	Gieseke <i>et al.</i> , 2001

^a used with unlabeled competitor as indicated in the reference

3. Results and Discussion

3.1 Decay experiments

The activated sludge model no. 3 (ASM3, Gujer *et al.*, 1999) was extended with two-step nitrification to enable separate modeling of ammonia and nitrite oxidation. Denitrification of nitrite and from nitrate to nitrite was not included. Loss of activity associated with loss of bacteria is modeled as one process (endogenous respiration) in the ASM3. Endogenous respiration of heterotrophic bacteria is a lumped process, which accounts for the oxygen utilization rate (OUR) due to growth on decay products, growth on very slowly degradable COD and probably also respiration of protozoa (Van Loosdrecht and Henze, 1999). In the following, heterotrophic OUR represents the base OUR measured before the addition of substrate (Figure 1: A), nitrification rates (OUR_{NOB}) after addition of nitrite (Figure 1: B) and nitrification rates (OUR_{AOB}) after addition of ammonia and complete enzyme synthesis (Figure 1: C). Effects of enzyme dynamics are discussed in chapter 3.2. Only graphical data of the experiments with CAS sludge are shown, experiments with MBR sludge yielded similar trends.

Aerobic conditions

Most activated sludge models (e.g. ASM3) summarize nitrification as one process. As a consequence, nitrite concentrations cannot be modeled and kinetics of AOB and NOB are lumped into one parameter set. However, elevated effluent concentrations of nitrite may occur causing problems in the receiving water due to its toxicity. In that case, a model extension with two-step nitrification is required involving the separate determination of the kinetics of AOB and NOB. Our previous research with activated sludge from the separate treatment of digester liquid suggested that the activity of NOB may decline faster compared to AOB (unpublished data).

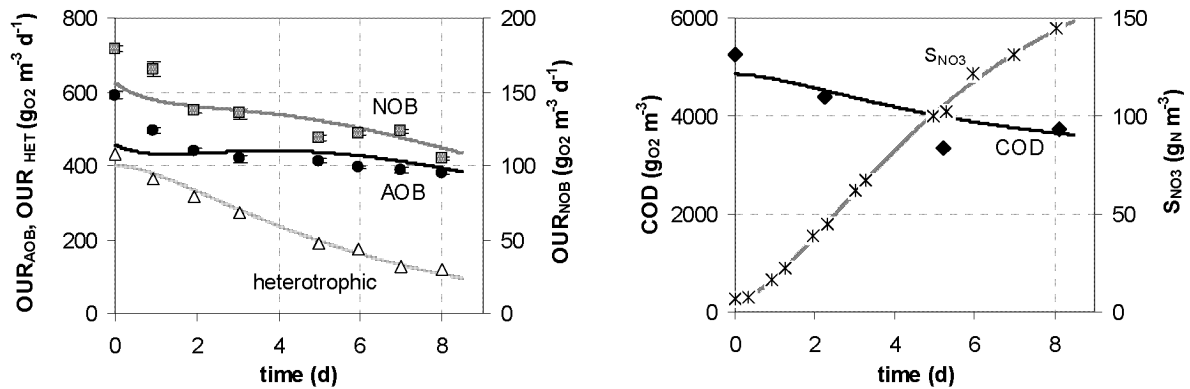


Figure 2 Results of batch experiment for CAS sludge under aerobic conditions at 20°C and pH 7.5. Lines represent best fit applying the adapted ASM3.

The results of the aerobic experiment for sludge from the CAS are shown in Figure 2. The activity is reduced to about 60% of the initial value after 7 days for both AOB and NOB. During the experiment, large amounts of organic nitrogen are released due to the endogenous res-

piration of heterotrophic bacteria, hydrolyzed to ammonium and immediately nitrified to nitrate. Since this study focuses on nitrifying bacteria, the heterotrophic endogenous respiration process in the ASM3 was slightly adapted in order to better fit the accumulation of nitrate. Therefore, a term considering the observed lag phase of the nitrate accumulation was introduced (Table 2) and the nitrogen content of bacteria was assumed to be 10% referring to COD.

Table 2 Adaptation of ASM3. X_H = heterotrophic bacteria. f_{lag} = fit parameter. t = simulation time, t_0 = initial time.

Process	Process rate equation ρ
Aerobic endogenous respiration of X_H	$b_{H,aer} \cdot \frac{S_O}{K_O + S_O} \cdot \frac{t - t_0}{f_{lag} + (t - t_0)} \cdot X_H$

Despite the fact that nitrate accumulation and subsequent growth of autotrophic bacteria on the released nitrogen is well reproduced by the adapted ASM3, the sharp loss of autotrophic activity during the first two days of the experiment seems not to be consistent with the underlying model processes. Thus, data points at $t = 0$ were omitted for the estimation of the parameter listed in Table 3. The values of the estimated autotrophic endogenous respiration rates correspond to literature data for one-step nitrification (summarized in Martinage and Paul, 2000). No differences between AOB and NOB were found. By contrast, measurements with sludge from the separate treatment of digester liquids resulted in a 50% higher rate for NOB than AOB confirming our previous observation (unpublished data). The aerobic heterotrophic endogenous respiration rates agreed well with values used for modeling in previous studies (Gujer *et al.*, 1999; Koch *et al.*, 2000). Experiments with sludge from the MBR yielded similar results.

Table 3 Estimated aerobic endogenous respiration rates (with standard errors) at 20°C using the adapted ASM3.

	AOB $b_{AOB,aerobic} (d^{-1})$	NOB $b_{NOB,aerobic} (d^{-1})$	heterotrophic $b_{HET,aerobic} (d^{-1})$
CAS	$0.15 \pm 0.02^{1)}$	$0.15 \pm 0.01^{1)}$	$0.28 \pm 0.05^{1)}$
MBR	0.14 ± 0.01	0.14 ± 0.01	0.23 ± 0.03

¹⁾ mean value from two experiments

The quantification of AOB using FISH confirmed the sharp reduction of activity in the beginning of the experiment (Figure 3). No shift within the population of AOB was observed during the experiment. This finding indicates that after two days a significant amount of AOB has either fallen into a dormant state (below detection limit for FISH) or grazed by protozoa. Predation of readily accessible bacteria in the beginning may be an explanation, although 10-20% percent of the overall autotrophic bacteria would have to belong to this group (Figure 2). Since sludge samples were taken from pilot plants running close to a steady state, such high percentages are rather unlikely. Moreover, the organization of AOB and NOB as dense aggre-

gates (Manser *et al.*, 2004; Wagner *et al.*, 1995) may additionally prevent autotrophic bacteria from major predation.

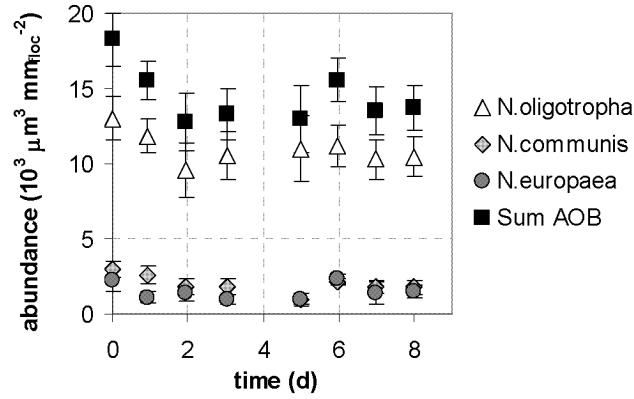


Figure 3 Quantification of ammonia-oxidizing bacteria (AOB) in the aerobic experiment using FISH.

Anoxic conditions

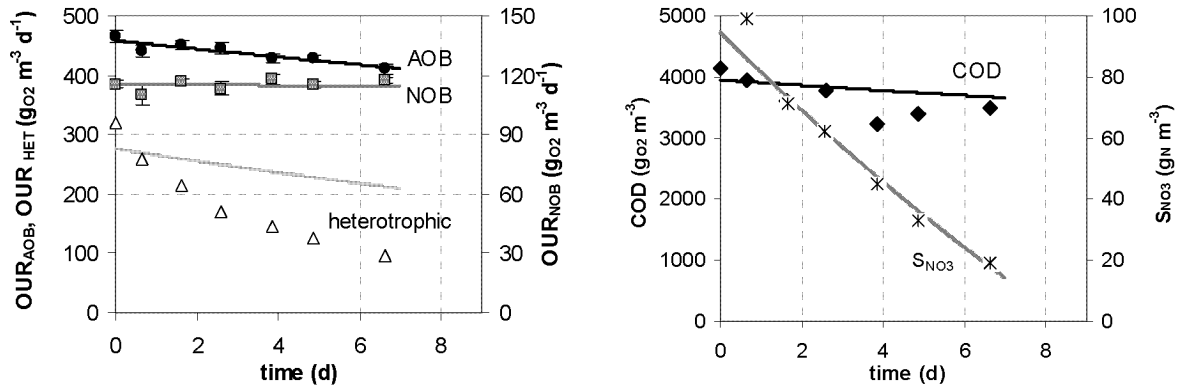


Figure 4 Results of batch experiment for CAS sludge under anoxic conditions at 20°C and pH 7.5. Lines represent best fit applying the ASM3.

The results of the anoxic experiment for sludge from the CAS are shown in Figure 4. Loss of activity is negligible for both AOB and NOB over a period of one week. Estimated anoxic endogenous respiration rates of autotrophic bacteria were 0.02 d^{-1} or lower for either CAS or MBR sludge (Table 4). By contrast, previous studies (Lee and Oleszkiewicz, 2003; Siegrist *et al.*, 1999) stated only 30-50% reduction of these parameters under anoxic compared to aerobic conditions. Inhibition effects associated with the accumulation of decay products in the batch reactors or the missing of short aerobic periods (enabling the nitrifiers to restore their ATP pool) may have led to a significant decrease of the autotrophic activity also at anoxic conditions. Likewise, very low heterotrophic endogenous respiration rates were estimated from the measured nitrate concentrations. However, the model clearly diverges from the measured heterotrophic OUR. The estimation of the anoxic endogenous respiration rates of heterotrophic bacteria based on the OUR_{HET} data would yield 0.16 d^{-1} and 0.13 d^{-1} for the CAS and MBR,

respectively. Other processes like decay of protozoa may have contributed to the observed decline of the heterotrophic OUR. Furthermore, the modeled heterotrophic OUR mainly depend on the estimated endogenous respiration rates from the aerobic experiments. While OUR due to heterotrophic endogenous respiration and predation is reasonably lumped into one parameter under aerobic conditions, this simplification may not reliably reproduce measured OUR under anoxic conditions.

Table 4 Estimated anoxic endogenous respiration rates (with standard errors) at 20°C using the ASM3.

	AOB	NOB	heterotrophic
	$b_{\text{AOB,anoxic}} \text{ (d}^{-1}\text{)}$	$b_{\text{NOB,anoxic}} \text{ (d}^{-1}\text{)}$	$b_{\text{HET,anoxic}} \text{ (d}^{-1}\text{)}$
CAS	0.015 ± 0.004	< 0.001	0.033 ± 0.002
MBR	0.01 ± 0.003	0.02 ± 0.009	0.064 ± 0.002

Alternating anoxic-aerobic conditions

Bacteria are exposed to alternating ORP conditions in wastewater treatment plants with nutrient removal. Since nitrifying bacteria are obligate aerobes, they are unable to store or utilize their substrate under anoxic conditions due to the lack of oxygen. As a result, stress and damage to their metabolisms may occur under anoxic conditions leading to increased substrate requirements during the aerobic period, also known as feast-famine phenomenon (Chen and Liu, 1999; Chen *et al.*, 2001).

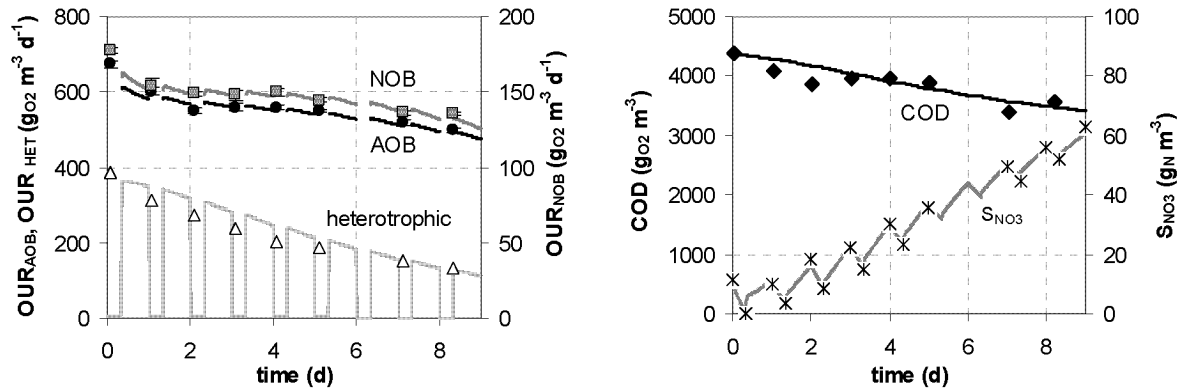


Figure 5 Results of batch experiment for CAS sludge under alternating anoxic-aerobic conditions at 20°C and pH 7.5. Lines represent best fit applying the adapted ASM3. Activity of AOB and NOB during anoxic periods was omitted for better readability.

The results of the alternating anoxic-aerobic experiment for sludge from the CAS are shown in Figure 5. Likewise to the aerobic experiment, the ASM3 had to be adapted (Table 2) in order to fit the accumulation of nitrate. In contrast to the completely aerobic experiment, the measured decrease of autotrophic activity could be reasonably modeled using the adapted ASM3 (Figure 5). The estimated aerobic endogenous respiration rates of AOB and NOB are shown in Table 5, assuming mean anoxic rates estimated from the anoxic experiment (0.01 d^{-1}). While endogenous respiration rates of AOB and heterotrophic bacteria from the

aerobic experiment (Table 3) were confirmed, aerobic endogenous respiration rates of NOB and anoxic endogenous respiration rates of heterotrophic bacteria had to be increased to better fit the measured data. The results suggest that NOB are more stressed under alternating anoxic-aerobic conditions than AOB. Increased grazing may be responsible for the elevated anoxic endogenous respiration rates of heterotrophic bacteria, because protozoa are known to survive short anoxic periods.

Table 5 Estimated endogenous respiration rates (with standard errors) from the anoxic-aerobic experiment at 20°C using the adapted ASM3.

	AOB		NOB		heterotrophic	
	$b_{AOB,aerobic} (d^{-1})$	$b_{AOB,anoxic} (d^{-1})$	$b_{NOB,aerobic} (d^{-1})$	$b_{NOB,anoxic} (d^{-1})$	$b_{HET,aerobic} (d^{-1})$	$b_{HET,anoxic} (d^{-1})$
CAS	0.15±0.01	0.01 ¹⁾	0.22±0.01	0.01 ¹⁾	0.27±0.02	0.06±0.004
MBR	0.13±0.01	0.01 ¹⁾	0.18±0.01	0.01 ¹⁾	0.23±0.02	0.10±0.01

¹⁾ mean value derived from anoxic experiment

In conclusion, no significant differences between conventional and membrane activated sludge were found indicating the same processes being responsible for the loss of autotrophic activity in the two systems. The feast-famine phenomenon resulting in a reduced decrease of microbial activity as previously reported (Lee and Oleszkiewicz, 2003) was not. Our experiments suggest that environmental conditions are crucial for the loss of microbial activity. However, from a microbiological point of view, it is likely that most bacteria do not die – unless they are exposed to e.g. toxic compounds –, they rather become dormant (Kaprelyants and Kell, 1996). Our observations fit the hypothesis that predation plays a major role in activated sludge systems (Van Loosdrecht and Henze, 1999), since protozoa are obligate aerobic and anoxic heterotrophic endogenous respiration rates are significantly reduced compared to the rates under aerobic conditions. In addition, the decline of the heterotrophic OUR is not consistent with the decrease of the denitrification activity under anoxic conditions. It remains however uncertain how protozoa affect nitrifiers, because the latter preferably grow in dense aggregates. The negligible loss of autotrophic activity under anoxic conditions is possibly rather due to the inhibited metabolism of the obligate aerobic nitrifying bacteria.

3.2 Enzyme dynamics

Enzyme processes (activation, synthesis, degradation, inhibition) are comparably fast and therefore cannot account for the loss of autotrophic activity in the beginning of the aerobic experiments. Nonetheless, enzyme dynamics can play a role for plants with fluctuating loads. Enzyme activation is the fastest mechanism to regulate a process and can be stopped or reactivated within minutes, depending on the presence of an inhibitor. In our measurements we found constant activation times of about 10 minutes for nitrification (Figure 6A). The subsequent enzyme synthesis (ammonia monooxygenase AMO and hydroxylamine oxidoreductase HAO) took between 1 and 2 hours, which is in agreement with literature data (Aoi *et al.*, 2004; SayavedraSoto *et al.*, 1996). By contrast, NOB immediately reached their maximum OUR. It is concluded that NOB do not or only marginally degrade their enzymes regardless of

the environmental conditions. The enzyme saturation for every measurement was calculated as ratio of the OUR after the activation time and the maximum OUR after complete enzyme synthesis. Cell growth during the phase of enzyme synthesis was neglected for this calculation. As shown in Figure 6B, enzymes are aerobically degraded to about 60% of their maximum level after 6 hours with a following only marginal decline. The continuous release of ammonia due to heterotrophic decay causes the AOB to keep a minimal level of the enzyme saturation. Similarly, half-life times on the order of hours were reported (SayavedraSoto *et al.*, 1996), whereas Aoi *et al.* (2004) could still detect one-third of the enzymes 24h after the start of starvation. Surprisingly, enzyme degradation was more enhanced at aerobic than at anoxic conditions, although AOB were able to continuously nitrify small amounts of released ammonia (Figure 6C). Despite the fact that degradation of enzymes basically takes place regardless of environmental conditions, anoxic conditions seem to prevent a significant decrease of the enzyme saturation in AOB.

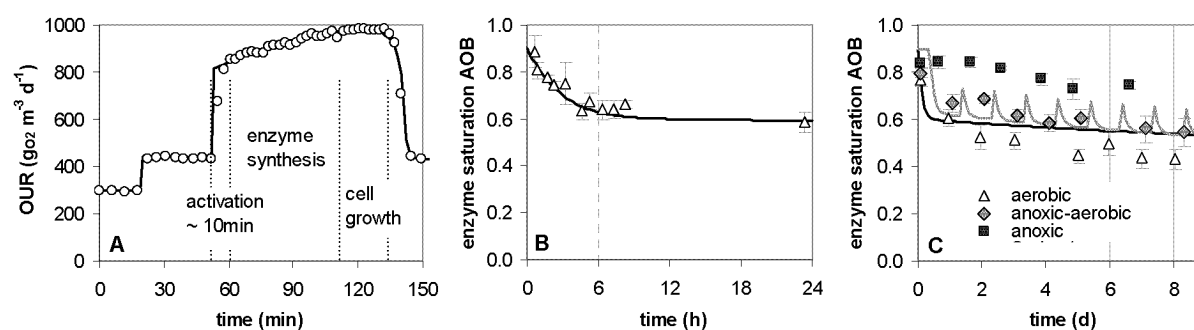


Figure 6 Illustration of enzyme activation and synthesis (AOB) for one measurement (A). Short-term degradation of enzymes (AOB) at aerobic conditions (B). Long-term degradation of enzymes (AOB) during decay experiments (C). The enzyme saturation was calculated as ratio of the OUR after the activation time and the maximum OUR after complete enzyme synthesis. Lines represent best fit using the ASM3 extended with enzyme kinetics (Table 6). Data from experiments with conventional activated sludge are shown.

The ASM3 (with already implemented two-step nitrification) was extended with enzyme kinetics adapted from Wild *et al.* (1995) in order to test its sensitivity on ammonia and nitrite dynamics in wastewater treatment systems (Table 6).

Table 6 Stoichiometric matrix and process rate equations (adapted from Wild *et al.*, 1995). E_{sat} = Enzyme saturation (-).

Process	E_{sat}	Process rate (ρ)
Enzyme synthesis of X_{AOB}	1	$k_{\text{synth}} \cdot \frac{S_{\text{NH}}}{K_{\text{NH, AOB}} + S_{\text{NH}}} \cdot \frac{S_{\text{O}}}{K_{\text{O, AOB}} + S_{\text{O}}} \cdot \frac{S_{\text{ALK}}}{K_{\text{ALK, AOB}} + S_{\text{ALK}}} \cdot (1 - E_{\text{sat}})$
Aerobic enzyme decay of X_{AOB}	-1	$k_{\text{decay}} \cdot \frac{S_{\text{O}}}{K_{\text{O, AOB}} + S_{\text{O}}} \cdot E_{\text{sat}}$
Growth of X_{AOB}	-	$\mu_{\text{m, AOB}} \cdot \frac{S_{\text{NH}}}{K_{\text{NH, AOB}} + S_{\text{NH}}} \cdot \frac{S_{\text{O}}}{K_{\text{O, AOB}} + S_{\text{O}}} \cdot \frac{S_{\text{ALK}}}{K_{\text{ALK, AOB}} + S_{\text{ALK}}} \cdot X_{\text{AOB}} \cdot E_{\text{sat}}$

In ASM3, the growth rate of X_{AOB} has to be multiplied by the degree of cell saturation with nitrification enzymes. E_{sat} is proportional to the actual total amount of enzymes divided by the actual biomass of X_{AOB} . It is assumed that anoxic decay of nitrification enzymes is negligible. The two new processes allow reasonably reproducing the measured values (Figure 6). Only the long-term degradation during the aerobic decay experiment is underestimated by the model (Figure 6C), which however should not play a role in practical operation of wastewater treatment plants. Data depicted in Figure 6B and several data sets from batch experiments (sample shown in Figure 6A) were used to estimate the unknown parameters k_{synth} and k_{decay} . The obtained values at 20°C are as follows:

$$k_{\text{synth}} = 30 \pm 4 \text{ d}^{-1} \quad k_{\text{decay}} = 3.0 \pm 0.3 \text{ d}^{-1}$$

The maximum enzyme saturation is lower than 1 due to the continuous decay of the enzymes. With the estimated parameters, the maximum achievable enzyme saturation is 0.9 at 20°C. The temperature dependency is assumed to be equal to the growth of AOB.

3.3 Significance for wastewater treatment

A simulation study was carried out in order to test the sensitivity of the reduction of anoxic autotrophic decay and the enzyme kinetics on the nitrification process. Three different model configurations based on the ASM3 with two-step nitrification were applied:

Model A: ASM3 default. Anoxic autotrophic decay = 50% of aerobic decay.

Model B: Anoxic decay = 0. No autotrophic decay at anoxic conditions.

Model C: Including enzyme kinetics. Model B extended with enzyme kinetics for AOB (Table 6). Maximum growth rate of AOB ($\mu_{\text{m, AOB}}$) was increased by factor $1/E_{\text{sat, max}} = 0.88$ (Figure 8) in order to exclude the effect of an overall reduced growth rate.

A CAS with three completely mixed reactors was assumed. The hydraulic retention time was chosen to be 12h. Temperature was set to 15°C. A typical diurnal influent variation of the ammonia load for municipal wastewater was used (Figure 7).

The sensitivity of the model predictions was investigated on two common plant layouts (Figure 7): fully aerobic and with 33% anoxic volume. The dissolved oxygen concentration in the aerobic reactors was fixed at $2.5 \text{ gO}_2 \text{ m}^{-3}$, whereas the aerobic sludge age was set to 6d in both layouts.

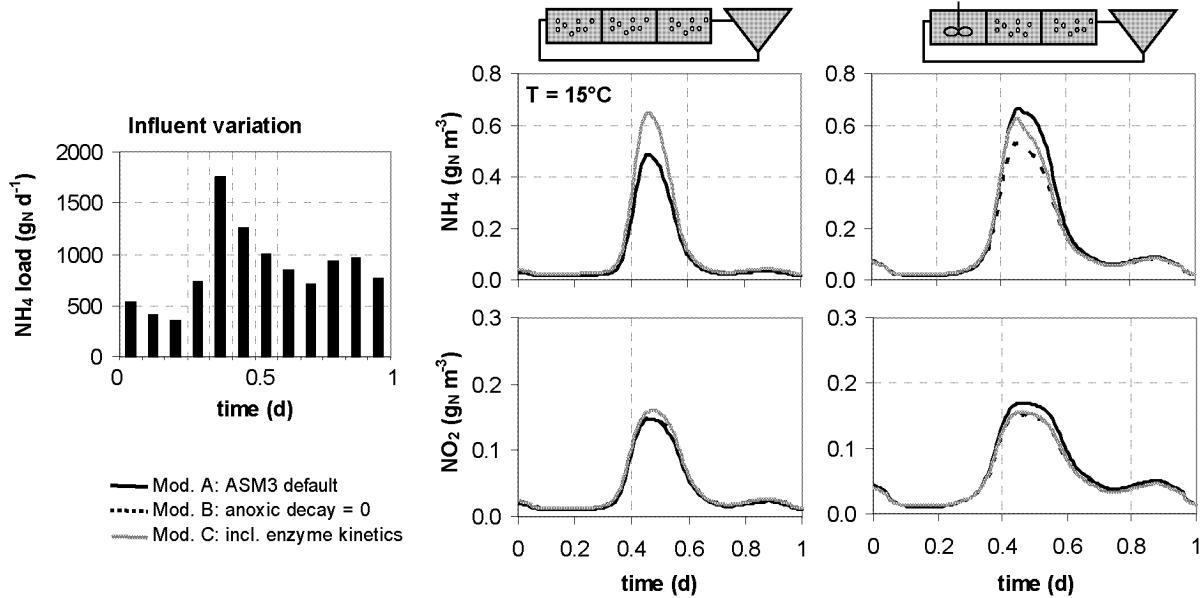


Figure 7 Simulated concentrations of ammonia and nitrite in the effluent of the third reactors.

The results reveal that enzyme kinetics has an impact on the ammonia concentrations in the effluent for typical diurnal variation in the influent. Neglecting enzyme dynamics leads to an underestimation of the ammonia concentrations for fully aerobic and partly anoxic plants. However, the differences are not large and could be compensated by e.g. a 10% increase of the maximum growth rate of AOB. Pre-denitrification obviously decreases the nitrification performance in Model A due to loss of nitrifiers within the anoxic reactor, whereas Model B (without anoxic autotrophic decay) predicts equal ammonia effluent concentrations compared to the fully aerobic plant layout. Similarly, anoxic conditions diminish the diurnal variation of the enzyme saturation of AOB (Figure 8, only aerobic degradation of enzymes assumed) leading to a slightly lower ammonia peak in the effluent (Figure 7). Nonetheless, the enzyme saturation is always above 75% even at fully aerobic conditions for the chosen domestic influent characteristics. Interestingly, the combined effect of reduced anoxic decay and enzyme kinetics (Model C) results in almost identical ammonia effluent concentrations compared to the default ASM3 (Model A) for the plant with pre-denitrification. By contrast, effluent nitrite concentrations are barely influenced by neither anoxic decay nor enzyme dynamics of AOB.

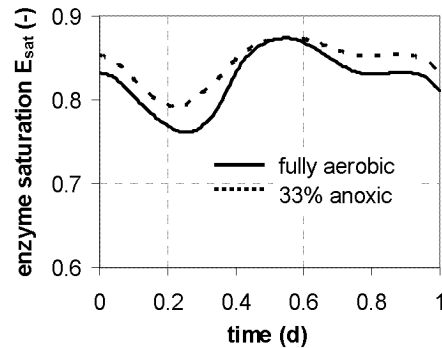


Figure 8 Diurnal variation of enzyme saturation E_{sat} of AOB (Model C).

4. Conclusions

Batch experiments using activated sludge from a conventional and a membrane bioreactor pilot plant were performed in order to study the decay of ammonia- and nitrite-oxidizing bacteria. It was found that:

1. Loss of autotrophic activity was only partly explained by the endogenous respiration concept as incorporated e.g. in the activated sludge model no. 3 (ASM3). Other processes like predation by protozoa may play a significant role. Furthermore, it must be considered that activated sludge samples were taken from continuous flow systems and analyzed in batch systems, where possibly other mechanisms occur.
2. The activity of both ammonia- and nitrite-oxidizing bacteria incubated at anoxic conditions did not decrease over several days. This must be considered when modeling wastewater treatment plants with anoxic volume. Otherwise, nitrification performance will be underestimated. An additional influence of alternating anoxic-aerobic conditions on the autotrophic decay rates due to the feast-famine phenomenon was not detected.
3. Ammonia- and nitrite-oxidizing bacteria exhibited similar endogenous respiration rates. Likewise, no differences were found between the conventional and the membrane activated sludge suggesting the same processes being responsible for loss of activity in the two systems. Therefore, decay parameters estimated from conventional systems can also be applied to membrane bioreactors.
4. Unlike nitrite-oxidizing bacteria, ammonia-oxidizing bacteria showed a pronounced decay of their enzymes. The extension of activated sludge models with enzyme kinetics for ammonia-oxidizing bacteria may lead to a better understanding of ammonia dynamics in wastewater treatment plants.

Further research is needed to elucidate the processes involved in the loss of autotrophic activity in activated sludge systems. In particular, in situ measurement of the decay of e.g. radio-labeled nitrifiers in continuous flow systems would avoid the change to batch conditions.

Conclusions

Conclusions

The developed method based on fluorescence *in situ* hybridization (FISH) and epifluorescence microscopy facilitates a reliable and fast **identification and quantification of nitrifying bacteria** in activated sludge. A comprehensive set of oligonucleotide probes targeting different nitrifying groups is available allowing for a detailed analysis of the nitrifying community composition. Although FISH and epifluorescence microscopy is still a tool mostly used for research, operators of large wastewater treatment plants may apply this technique for monitoring purposes. Thus, nitrification failures due to inhibition or wash-out of the nitrifiers could be detected or mitigation measures against sludge bulking and foaming could be optimized based on the analysis of filamentous bacteria.

The membrane separation does not influence the **nitrifying community composition** but has a dramatic impact on the floc structure. Therefore, MBRs operated at non-elevated sludge ages (10-30d) are expected to harbor the same bacterial groups compared to CAS exhibiting equal physiological properties. Apart the microbial conversions, other processes such as mass transfer effects play a role in activated sludge systems and are implicitly considered in lumped **model parameters**. However, mass transfer limitation is negligible in MBRs due to their small floc size compared to CAS. Consequently, MBRs can be operated at low oxygen concentrations in order to save aeration energy and to improve pre-denitrification.

Likewise, **decay** is not affected by the membrane separation. It however strongly depends on the oxidation-reduction potential. The activity of both ammonia- and nitrite-oxidizing bacteria was only marginally reduced under anoxic conditions, whereas aerobic conditions similarly diminished their activity. As a consequence, WWTPs extensions of anoxic volumes or control strategies optimizing aerated volumes enhance nitrification capacity in terms of remaining nitrifiers rather than reduce it.

The enriched community composition is crucial for success and failure of **bioaugmentation**. WWTPs harbor nitrifiers, which are well adapted to low substrate concentrations (mostly *Nitrosomonas oligotropha* and *Nitrospira*), whereas ammonium-rich influent like sludge digester liquids lead to a nitrifying community with high maximum growth rates but low affinities to the substrates (mostly *Nitrosomonas europaea* and *Nitrobacter*). Since the latter group is not able to adapt to low substrate levels within days, bioaugmentation using enriched sludge from the separate treatment of digester liquids is not necessarily suited for the long-term enhancement of the nitrification performance. For this reason, the complete retention of bacteria due to the membrane separation does not play a role in this context.

Activated sludge models used for CAS can reliably be applied to MBRs in terms of model structure. However, kinetic parameters implicitly considering mass transfer effects - such as half-saturation constants for oxygen - have to be adapted. For CAS exhibiting large flocs and a high influent variation, dynamic (depending on the active processes) half-saturation constants mimicking mass transfer effects may improve the quality of the model. A more detailed model of ammonia oxidation must include enzyme kinetics, since ammonia-oxidizing bacteria rapidly degrade their enzymes unlike nitrite-oxidizing bacteria.

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