# TOWARDS THE INTEGRATION OF OXIDATIVE AND REDUCTIVE ACTIVITIES: APPLICATION TO NITROGEN REMOVAL BY CO-IMMOBILIZED MICROORGANISMS

**Vítor Alexandre Pires Martins dos Santos**

Promotor: Prof. dr. ir. J. Tramper
Hoogleraar in de Bioprocestechnologie

Co-promotor: Dr. ir. R. H. Wijffels
Universitair docent bij het departement Agrotechnologie en Voedingswetenschappen, sectie Proceskunde

Samenstelling promotiecomissie:

Prof. dr. ir. M.C.M. van Loosdrecht, Technische Universiteit Delft
Prof. dr. ir. R.M. Boom, Wageningen Universiteit
Dr. ir. C.Vilchez-Lobato, Universidad de Huelva
Dr. ir. A. J. M. Stams, Wageningen Universiteit
Dr. ir. D. de Beer, Max-Planck Institut Bremen

Vítor Alexandre Pires Martins dos Santos

# TOWARDS THE INTEGRATION OF OXIDATIVE AND REDUCTIVE ACTIVITIES: APPLICATION TO NITROGEN REMOVAL BY CO-IMMOBILIZED MICROORGANISMS

**Proefschrijft**

**ter verkrijging van de graad van doctor op gezag van de rector magnificus van de Landbouwuniversiteit Wageningen,**

**prof. dr. ir. L. Speelman**

**in het openbaar te verdedingen**

**op dinsdag 3 april 2001**

**des namiddags te 13.30**

**in de aula van de Universiteit Wageningen**

16 11 02

Het onderzoek beschreven in dit proefschrift werd uitgevoerd bij de sectie Proceskunde, departement Agrotechnologie en Voedingswetenschappen, Wageningen Universiteit.

This work was financially supported by the Junta Nacional de Investigação Científica e Tecnológica (JNICT), Portugal (BD/1505/91 IF and BD/3753/94).

Cover: Old photograph (1920-1930) of a channel crossing the centre of Aveiro, Portugal. The people in the boat, a „Moliceiro", are collecting "moliço" (algae and submerged vascular plants) for its later use as a fertiliser. The "moliço" accumulate in the channels and river deltas of this lagunar region due to the excess of nitrogen and phosphates (eutrophication). By Henrique Ramos, Aveiro, Portugal.

Printing: Ponsen & Looijen B.V., Wageningen, The Netherlands

Martins dos Santos, Vítor Alexandre Pires

Towards the integration of oxidative and reductive activities: application to nitrogen removal by co-immobilized microorganisms

Thesis Wageningen University , The Netherlands – With references – With summary in Duch and English – 354 pp

**ISBN – 90-5808-390-X**

Subject headings: (de)nitrification, (co-)immobilized cells, dynamic modelling, microelectrodes, air-lift reactor, double-layer beads, shear, rheology

Aos meus pais Maria Elisa e Vítor

Em memória do meu irmão Carlos Filipe

Para a minha mulher Maria del Carmen

MAT 01.2955

**Stellingen**

1. Kunstmatig geïmmobiliseerde celsystemen zijn uiterst geschikt om het dynamisch gedrag van geïmmobiliseerde micro-organismen in het algemeen te bestuderen.

2. Een volledige ontwikkeling van wiskundige en theoretische modellen die inzicht verschaffen in complexe biologische processen is alleen mogelijk als de ontwikkeling van experimentele technieken die dergelijke modellen en theorieën kunnen toetsen gelijke tred houdt.

3. Goede experimenten zijn alleen mogelijk op basis van een goed doordachte theorie. Zelfs serendipiteit komt vrijwel altijd voort uit goed doordachte experimenten die anders verlopen dan men dacht.

4. Onderzoekers kunnen veel leren van menige microbiële gemeenschap aangaande effectieve samenwerking in multidisciplinaire wetenschappen.

5. Het publiceren van artikelen in tijdschriften als "Annual Review of Failed Research", "Trends in Wishful Thinking", "Critical Reviews in Nice-But-Does-Not-Work Ideas", or "Journal of Irreproducible Research", zou zeker helpen het aantal onnodige herhalingen van fouten en het inslaan van doodlopende wegen te voorkomen, en zou dus een belangrijk bijdrage aan de ontwikkeling van de wetenschap in het algemeen leveren.

6. Het bewust verlaten van een bepaalde onderzoeksrichting of zelfs vakgebied naar een andere richting of vakgebied vergroot de kans op innovatie.

7. Ondanks een steeds meer gecoördineerd economisch, sociaal en politiek beleid van de EU-staten, die min of meer naar een Verenigd Europa met behoud van eigen identiteit streven, heeft menig EU-burger een zeer beperkte kennis over zijn mede EU-burgers. Dit bemoeilijkt begrip van eigen identiteit zodanig dat het werkelijke convergentie in de weg staat.

8. Bovenstaande endemische culturele kortzichtigheid zou actief gecorrigeerd moeten worden door een goed doordacht en consistent beleid van EU en nationale overheden. Mogelijke voorbeelden hiervan zijn verplichte uitwisseling van scholieren of studenten tussen de verschillende EU-landen en vakken op school die zich specifiek met Europese cultuur bezig houden.

Stellingen behorende bij het proefschrift "Towards the integration of oxidative and reductive activities: application to nitrogen removal by co-immobilized microorganisms" door Vítor Alexandre Pires Martins dos Santos.

Braunschweig, 3 april 2001

# VOORWOORD

Eindelijk is het af!!! Toen ik in 1996 Nederland verliet, had ik het meeste van dit proefschrift eigenlijk al geschreven. Ik dacht toen dat het mij nog zo'n 3 á 4 maanden zou kosten om de boel af te ronden als ik in Nederland zou blijven. Ik verwachtte dat als ik verder van Nederland vandaan zou zijn het me ongeveer twee maal zoveel tijd zou gaan kosten. Fout! Het heeft me nog ruim vier jaar gekost om even tussen door iets op papier te zetten. Ik ben dus (evenals mijn promotor en co-promotor, mijn vrouw en mijn ouders) zeer opgelucht dat het eindelijk zover is.

Zoals altijd het geval is, zijn er veel mensen die een belangrijke bijdrage hebben geleverd aan de totstandkoming van dit proefschrift. Nu is het zeker tijd, en waarschijnlijk de beste gelegenheid, om ze te bedanken.

Ten eerste wil ik zeer hartelijk Hans Tramper en René Wijffels (respectievelijk promotor en co-promotor) bedanken voor hun onuitputtelijke inspanning en steun, niet alleen gedurende de tijd van mijn promotie onderzoek zelf (waar hun kritische en constructieve houding zeer leerzaam en bepalend voor de afloop van het onderzoek zijn geweest) maar zeker ook gedurende de laatste 4 sukkelende schrijfjaren. In die tijd zijn ze gemiddeld meer dan een keer per jaar bij mij op bezoek geweest in verschillende landen om zeker te zijn dat ik door zou zetten. Dat heb ik enorm gewaardeerd en ik dank ze daar hartelijk voor. Bovendien was het gewoon heel erg leuk om met Hans of René in Granada of Braunschweig op stap te gaan. Ik hoop dat dergelijke bezoeken herhaald kunnen worden!

Voor dit proefschrift zijn er verschillende stukken onderzoek in nauwe samenwerking met mensen van verschillende instellingen uitgevoerd. Het werk in hoofdstukken 5 en 6 werd gedaan in een zeer prettige samenwerking met mijn collega Imke Leenen (Sectie Proceskunde van de Wageningen Universiteit) en Katja Grolle en Ton van Vliet (Leerstoelgroep Fysica en Fysische Chemie van Levensmiddelen van de WU). De toepassingen van het Toverbal Concept voor volledige autotrofe (de)nitrificatie met Anammox (hoodstuk 10 en 11) werd uitgevoerd in samenwerking met Mike Jetten en Gijs Kuenen van de Technische Universiteit Delft (Kluyver Laboratorium). De vele metingen van hoofdstukken 8 en 9 werden verricht aan de Universiteit van Amsterdam in het kader van een samenwerking met Peter Verschuren en Han van den Heuvel (Vakgroep Bioproceskunde). De nauwe samenwerking met Peter Verschuren heb ik als bijzondere plezierig ervaren. De lange dagen en avonden die wij spendeerden aan het kijken naar omhoog- of omlaaggaande kleine rode cijfertjes zal ik nooit vergeten!

Er zijn een aantal studenten die aan dit onderzoek meegewerkt hebben en wie een essentiële bijdrage hebben geleverd. Deze zijn (op volgorde van aankomst): Marjet Bruijnse, Jean-Paul Haerkens, Tatjana Vasilevska, Boglarka Kajuk, Leon Marchal, Mohammad Ali Hassan, Mireia Ripoll-Martinez, Catrinus van der Sluis, Harald Ruijssenaars, Herman Krab, Marc Jacobs, Alison Holland. Aan jullie allen: hartelijk bedankt.

De goede adviezen, theoretische en praktische hulp van Jan Hunik tijdens zijn laatste periode bij Proceskunde (en tegelijkertijd de begintijd van mijn promotieonderzoek) zijn essentiële geweest voor het gehele onderzoek. Dank ook aan Lesley Robertson van de Technische Universiteit Delft (Kluyver Laboratorium) voor het beschikbaar stellen van de denitrificerende cellen en voor goede adviezen. Mijn dank aan Wim Roelofsen van de vakgroep Microbiologie voor de hulp bij het opzetten van de chromatografische methoden. De deskundige adviezen van Ido Wolters (Proceskunde) en Gert Nieuweboer (Glasinstrumentmakerij) en van de medewerkers van de werkplaats zijn essentieel geweest voor het ontwerp van de airlift reactoren waarmee het meest van dit onderzoek uitgevoerd werd en die nog steeds gebruikt worden in andere onderzoeksgebieden bij Proceskunde. Kees de Gooijer, Fred van den End, Marcel Zwietering en Klaas van't Riet zijn altijd bereid geweest om over bepaalde aspecten van het onderzoek te discussieren.

Mijn (ex-) collega's bij Proceskunde hebben gezorgd voor een zeer plezierige en stimulerende sfeer zowel op professioneel en persoonlijk vlak. Behalve voor zijn steun tijdens de periode van dit onderzoek, dank ik mijn (ex-) kamergenoot en goede vriend Jaap Oostra voor zijn hulp met de correcte vertaling van de samenvatting. In het bijzondere herinner ik me met zeer veel plezier het „eventjes in Loburg een biertje drinken en een sigaartje roken" met Jaap op weg naar huis. Het was altijd erg gezellig en nooit „eventjes".

Agradeço e dedico esta tese aos meus pais que desde sempre me apoiaram incondicionalmente e que proporcionaram todas as condições que me permitiram fazer o que sempre gostei de fazer.

También quiero expresar mi especial agradecimiento hacia María del Carmen, mi mujer, cuyo apoyo ha sido fundamental para llevar a término esta tesis. Nunca le podré agradecer bastante el enorme apoyo que me ha dado y la paciencia que ha tenido, especialmente por aquellos momentos que no pude compartir con ella.

## CONTENTS

# CHAPTER 1

## Scope, Aims and Outline

### Background

Complete degradation of many pollutants requires sequenced anaerobic-aerobic biotreatment steps. Many compounds that are difficult to degrade aerobically are readily biotransformed anaerobically. The products of anaerobic biotransformation, however, will frequently resist to further mineralization; yet, they will be good substrates for aerobic biodegradation. Examples of this are the sequential biodegradation of highly chlorinated aromatics and aliphatics, azo-dyes, TNT, inorganic nitrogen compounds ($NH_4^+$, $NO_2^-$ and $NO_3^-$) and pesticides such as DDT, HCH's or methoxychlor. In waste- and groundwater treatment, these sequenced biotransformations are commonly achieved either by using aerobic and anaerobic (anoxic) reactors in series or by alternating periods of aerobiosis and anaerobiosis in a treatment unit. Ideally, however, these biodegradation processes would take place in a single, compact continuous-reactor system under carefully controlled conditions. In many instances, the benefits of such an integrated system would be clearly greater than the mere sum of the advantages of each individual process.

### Magic beads: an advanced engineering concept for process integration

This work addresses the possibilities of integrating oxidative and reductive complementary biodegradation processes in compact systems by using co-immobilized mixed-culture systems. The central idea throughout the book is that aerobic and anaerobic niches will eventually develop and coexist within a single biocatalytic particle so that oxidative and reductive activities (e.g nitrification and denitrification, respectively) can be accomplished simultaneously (Magic-beads). Therefore, multiple-step complementary biodegradation and biotransformation processes could be conducted as single staged (Figure 1). The rationale behind this idea relies on sound experimental evidence (e.g. time-dependent measurements of oxygen gradients across biocatalyst particles or biofilms) that shows that such niches do indeed establish under aerobic process conditions.

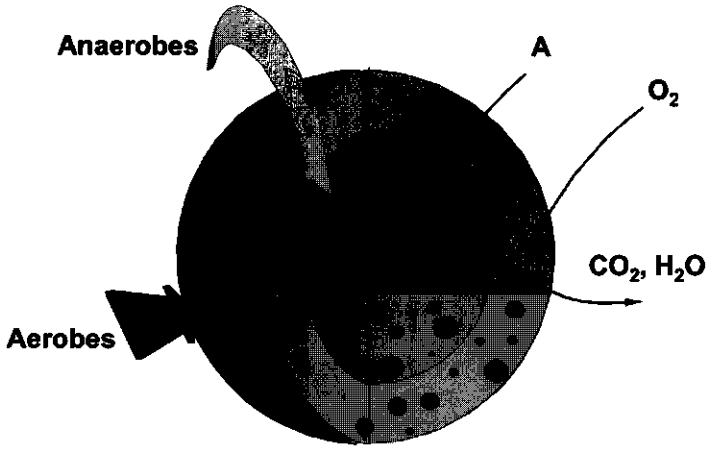


Figure 1 – Schematic representation of the "Magic-bead Concept".

**Case study: Integrated nitrification and denitrification**

The potential of the general concept outlined above for combining oxidative and reductive processes with relevance to the biodegadation of recalcitrant compounds is assessed in this work by studying in detail coupled nitrification and denitrification within (double-layered) gel beads for high-rate removal of nitrogen from wastewaters.

In such beads, the nitrifying microorganisms (aerobes) immobilized in an outer layer would oxidize ammonium into nitrite that would then diffuse inwards, where immobilized denitrifiers (either facultative heterotrophs or obligate anaerobic ammonium oxidizers) would reduce this nitrite into the harmless gaseous nitrogen. The biocatalyst particle is used optimally because both the external layers and core are active. The beads are placed in a common airlift reactor through which the waste streams can flow at almost any rate, without the need of recirculation to or from any anoxic compartment or reactor.

**Aims of the dissertation and outline**

This research project aimed at a) the development and characterization of a coupled system for integrated nitrogen removal, b) understanding the mechanisms underlying the processes involved and; c) providing knowledge for the integration of oxidative and reductive activities in a single compact system. With these aims in mind, the stepwise strategy depicted in Figure 2 was developed. Every stage of the project was comprised by a series of self-contained studies addressing different aspects of the proposed system.

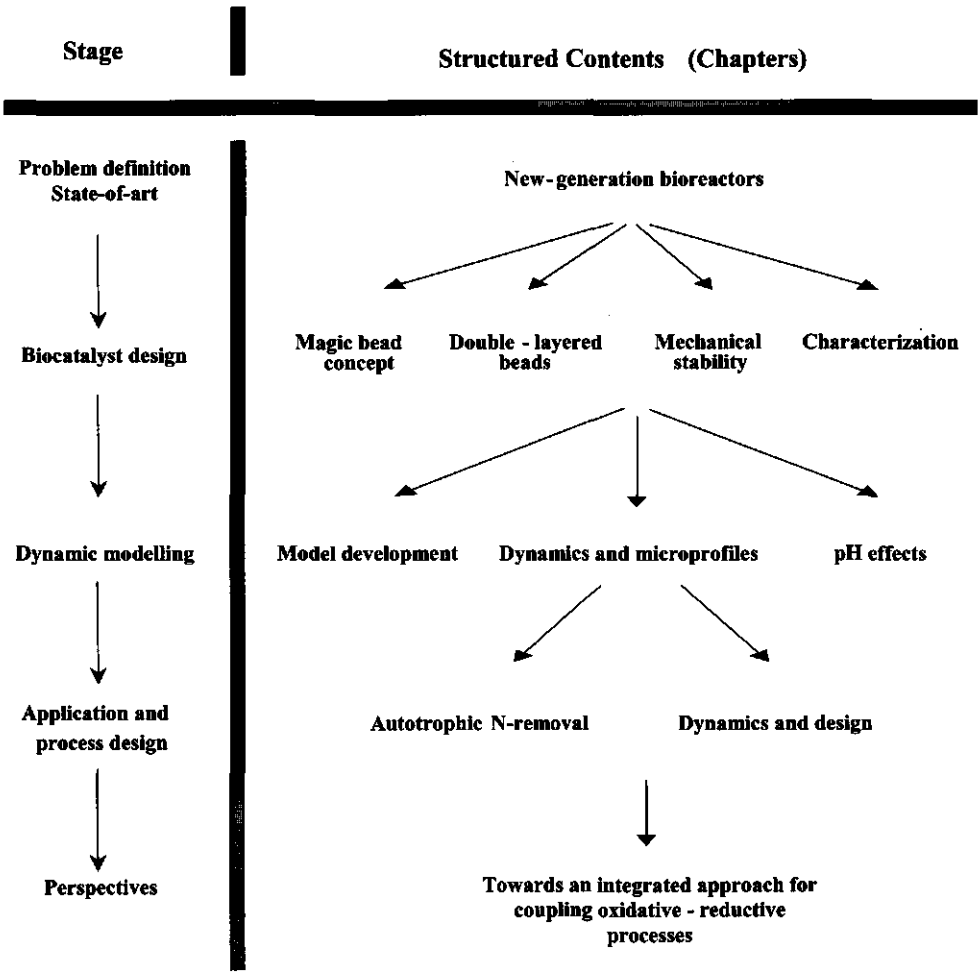


Figure 2 – Structured contents of this dissertation

In the first stage (chapter 2) the scope of the problem was defined (apparent incompatibility of oxidative and reductive activities of environmental relevance), the needs were addressed (urge to integrate processes and reduce reactor size) and the state-of-the-art of the field (conventional systems and emerging technologies) was presented. In the following phase (chapters 3 to 6), a compact process was proposed and the procedures for biocatalyst production, and its characterization and mechanical stability were assessed. In the next stage (chapter 7 to 9), achievement of in-depth insight into the system's behavior was pursued by means of mathematical modeling and

concomitant experimental validation using specific microlectrodes. The knowledge gathered up to this point was subsequently used successfully for the design of a fully autotrophic system for nitrogen removal (chapters 10 and 11). Finally, the possibilities of integrating other oxidative and reductive complementary biodegradation processes in compact systems by using co-immobilized mixed-culture systems were discussed in Chapter 12.

This chapter has been published in: Biotechnology Annual Review, 1998, 4:321-394 by Martins dos Santos VAP, Tramper J, Wijffels RH.

## CHAPTER 2

# Integrated Nitrogen Removal in Compact Systems by Immobilized Microorganisms: New-Generation Bioreactors

**ABSTRACT**

This paper gives an overview of the most recent advances on the development of compact systems for integrated nitrogen removal. First, a brief glance on the conventional configurations most commonly used is given. Compact systems based on immobilized-cell processes will be then discussed on basis of their performance, adequacy and potential applicability at large scale. Novel nitrification-denitrification processes will be evaluated as well as some important tools (such as modeling and its experimental evaluation by several ways) for the study of nitrogen removal in compact reactors. Finally, the future prospects on integrated nitrogen removal will be discussed.

The comparative analysis made of many advanced systems indicates that those that are the most compact and effective involve immobilized-cell technology (either naturally attached or artificially immobilized) and tend to be designed in height. This is just the opposite of the treatment plants currently in operation that are based chiefly in activated-sludge systems (suspended bacteria in large shallow tanks). Another important tendency is the attempt to integrate nitrification and denitrification within the most compact systems possible. The extreme cases are the systems in which both steps occur within the same biocatalyst particle. One of the most promising configurations found was that in which nitrogen removal proceeds fully autotrophically within a single carrier.

## INDEX

## Introduction

The intensification of industrial and agricultural practices, as well as the exponential growth of the human population and explosive urbanization in the last few decades have led to an enormous increase of the discharge of nitrogenous compounds in the environment. Severe environmental problems arise from these excessive loadings. Nitrogen compounds strongly enhance the eutrophication processes in closed-water areas and are thought to be associated (ammonia in particular) to algal blooms in lakes, rivers and coastal areas [1-3]. The discharge of gaseous nitrogen compounds in the atmosphere result in the formation of nitric and nitrous acids. Together with sulfuric acid, these compounds are the major constituents of acid rains, that cause corrosion of buildings [4], damage leafs and strongly contribute for acidification of soils and waters [1,5]. Other problems involve contamination of groundwater by nitrite and nitrate (that are thought to cause infantile methaemoglobinemia, [6-7]), greenhouse effects due to the accumulation of nitrous oxide in the atmosphere [8-10] and ozone loss due to formation of nitrate (and hence nitrous acid) from nitrite and ozone [11]. Looking at these problems it is not surprising that the environmental laws had become more and more strict towards the residual levels of nitrogen compounds allowed in wastestreams. According to the new E.C. directives on urban wastewater treatment [12], the effluents of sewage treatment plants (STP's) should not contain more than 15 mg/l total-N for STP's with design capacity of less than 20.000 p.e. (1 population equivalent, p.e. corresponds to 54 g $BOD_5$/head per day). For larger plants, this limit is 10 mg/l total-N, and in both nitrite should not exceed 1.0 mg./1. These values are similar to those

established by the U.S. Environmental Protection Agency [13] and to those demanded by the Environmental Water Quality Standards of Japan [14].

Nitrogen compounds are commonly removed within wastewater systems by suspended or flocculated biomass (activated-sludge) in reactors in series. Due to the slow growth rate of the microorganisms involved (especially of those responsible for oxidation of ammonia and nitrite), long retention times and large aeration tanks are required for effective nitrogen removal. To meet the sharpened limit-values for total-nitrogen in wastestreams even larger treatment units are needed. Frequently the space necessary to realize these extensions is not available at the treatment site. Therefore, intensive research and development work has been done in the last decades to design, build and operate high-rate, compact reactor systems for nitrification and denitrification with minimal space needs while meeting the environmental requirements.

This paper gives an overview of the most recent advances on the development of such compact systems for integrated nitrogen removal. Systems based on immobilized-cell processes will be discussed on basis of their performance, adequacy and potential applicability at large scale. Novel nitrification-denitrification processes will be evaluated as well as some important tools (such as modeling and its experimental evaluation by several ways) for the study of nitrogen removal in compact reactors. Finally, the future prospects on integrated nitrogen removal will be discussed.

**Demands on nitrogen removal**

The sharpening of the limit-values for total-nitrogen in wastestreams has important consequences for most of the nitrogen-removal systems currently in operation. As mentioned above, still a great majority of the existing nutrient-treatment plants are based on activated-sludge. To avoid the microorganisms of interest to be washed out of the reactor (and the slow-growing nitrifying cells in particular), the sludge is retained in the tanks for long time (typically 24-72 hours). This obviously limits the throughputs attainable in such systems, for which large (aerated) tanks are required. To meet the above-mentioned limit-values using activated sludge technologies even larger reactors are necessary. Frequently, the space required by these expansion is not available at the treatment site.

In addition to this, we emphasize that, despite many improvements, nitrification and denitrification still take place in two separate reactors or reactor compartments. It is thus clear that if both processes could be combined within a single, compact reactor system, much space and money could be saved. A straightforward advantage of combining these two processes in a single, compact system, is that two "superfluous" steps could be avoided. Indeed, in the nitrification process ammonia is first converted into nitrite

which is in turn oxidized into nitrate at expenses of oxygen. Subsequently, in the first denitrifying step nitrate is reconverted into nitrite while consuming organic carbon. By letting denitrification proceed directly from nitrite, about 25% oxygen and up to 40% organic carbon could be saved.

Thus clearly, there is a strong demand for new approaches in nitrogen removal technology that minimize the space requirements while meeting the strict environmental regulations. Table 1 summarizes the characteristics of activated-sludge systems and the future demands for wastewater treatment processes.

Table 1 - Negative aspects of activated-sludge processes and future demands*

| Negative aspect | Origin | Future demands |
|---|---|---|
| Large reactor volume | Low sludge concentration | Compact reactors system for treatment at the source |
| Large settler area | Low settling rate of biomass | Limited surface occupation |
| Low treatment efficiency | High sludge age required, low sludge loading | Increased efficiency |
| High surplus sludge production | Low sludge loading | Minimization of sludge production |
| "Double" space requirements | Separation of the nitrification and denitrification processes | Compact, single treatment system |
| "Superfluous" reactions | Separation of the nitrification and denitrification processes | Savings in energy and resources |
| High energy consumption | Extensive aeration in large nitrifying reactors | Minimization of energy consumption |
| Emissions of noise, odour and aerosols | Large open reactors | No burden on neighborhood |

* - adapted and extended , in modified form, from references [15-17].

In an attempt to improve the negative aspects shown in Table 1, much effort and investment has been done in developing more compact and effective systems. Figure 1 shows schematically the most important advances in the last decade towards improved processes.

The processes depicted represent a large part of the "newly developed" reactor units for nitrification and denitrification. For integrated nitrogen removal such units have often to be combined with one another. For instance, a denitrifying fluidized bed can be placed before a nitrifying air-lift whose effluent is partly recirculated to the fluidized bed.

These and other configurations will be discussed in more detail in the next sections. Mixed cultures processes refer here to reactor systems (hybrid reactors) in which

suspended biomass is deliberated made to coexist with biomass immobilized in a carrier. This will also be addressed later.

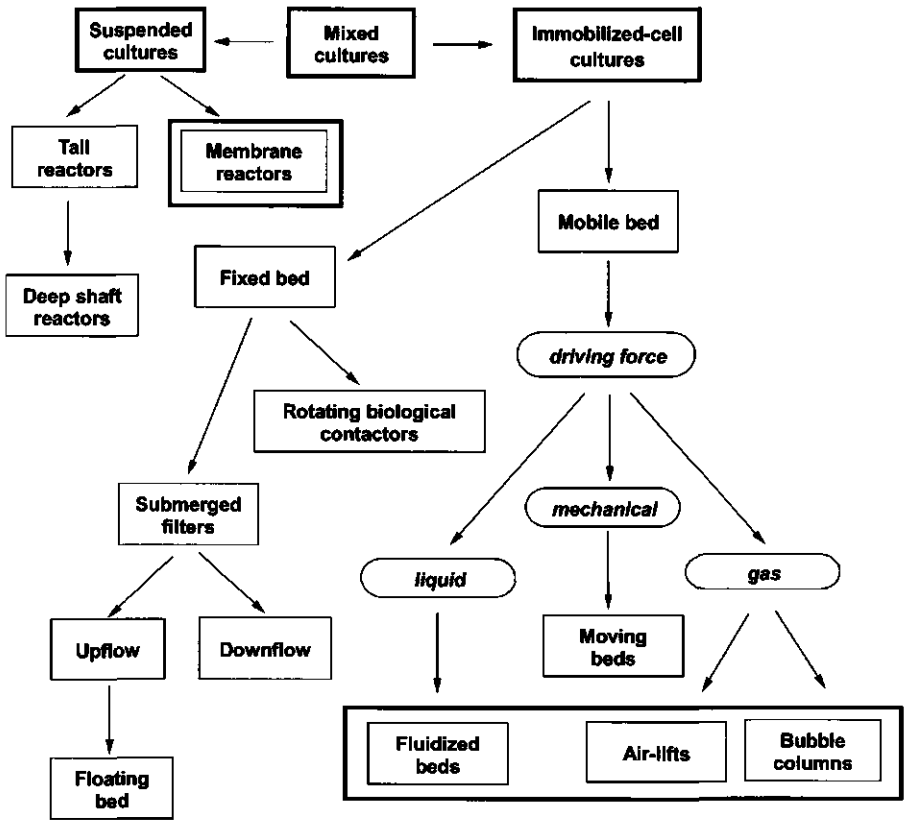


Figure 1 - Classification of advanced biological nitrogen removal processes according to Lazarova & Manem [18]. ▭ Represents reactor units that will be treated in detail in this review.

The classification procedure followed intends only to provide a given structure in the multitude of systems available. Thus obviously, definitions are not rigid. For instance, deep-shaft reactors (originally developed for suspended biomass) may be extended for operation with immobilized-cells if it is considered relevant. The scheme should be thus viewed as a flexible structure.

**Immobilized-cell processes**

It is clear from Table 1 that the low biomass concentrations attainable in activated-sludge systems strongly limits their treatment capacity and effectiveness. A rational way of circumventing this limitation is by concentrating biomass in the treatment unit. This can be done either by immobilization of the pertinent microorganisms onto (or within) solid carriers. Immobilization can be defined as the physical confinement of cells to a certain defined region of space while maintaining a given desired activity [19-20]. Immobilized cells can be divided into "naturally" attached cells (biofilms) and artificially immobilized cells [21]. By immobilization, the residence time of the liquid phase of a continuous-flow reactor can be uncoupled from the growth rate of the intervening biomass. As a result, much higher biomass concentrations can be attained in immobilized-cell reactors than in activated-sludge systems [17, 21-23] Therefore, higher capacities can be reached and smaller reactors can be used, with the additional advantages that cells are much easier separated from the treated effluent and that its concentration is no longer dependent on the efficiency of the sedimentation or centrifugation step.

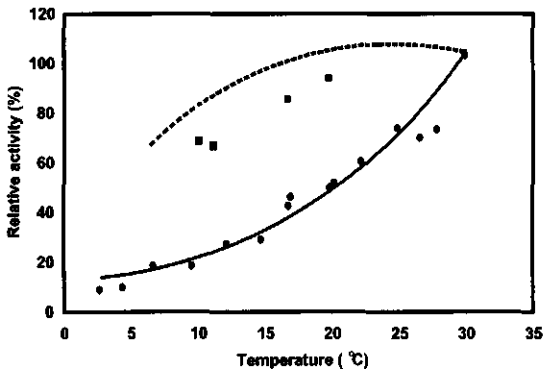


Figure 2 - Effects of immobilization of typical nitrifying cells on their nitrite-oxidizing capacity as a function of temperature (adapted from reference [24]).

Also, several studies have demonstrated that, under numerous conditions, immobilized-cells processes have other advantages over free-cell systems. Examples are the reduced sensitivity to lower temperatures [21, 24-29] (see also Figure 2) and increased tolerance to inhibitory agents [30-33] in comparison to suspended cells. These reduced sensitivities to external conditions seem to be related not to a real protective effect but rather to higher penetration depth of the limiting substrates into the layers of biomass as a result of a decrease in the activity of the biomass closer to the surface of the carriers

particle. The inner biomass plays thus an important role as "biomass buffer" that allows the system to face better adverse conditions.

*Naturally attached biomass (biofilms)*

The simpler way of immobilizing cells is by letting nature work. Under the right conditions (e.g., dilution rate in a reactor exceeding $\mu_{max}$ of the cells [34,35]), most microorganisms will easily attach to a solid (preferably rough) surface, which can be either a fixed substratum or a carrier, and form a biofilm, which is a complex structure of cells and cellular products (such as extracellular polysaccharides) bound to each other and to the support [35, 36-40]. Microorganisms attach as a way of protecting themselves from the starvation that would result of their washout of a given nutrient-rich environment [17,35,38]. Figure 3 represents schematically the process of biofilm development and detachment.

A very wide range of support materials is commonly used for biofilm processes. These supports (often spherically or cylindrically-shaped and preferably rough and cheap) include sand, basalt, wood chips, gravel, clay, activated carbon, pumice stone, silicates, vermiculite, stainless steel and ceramics, among others.

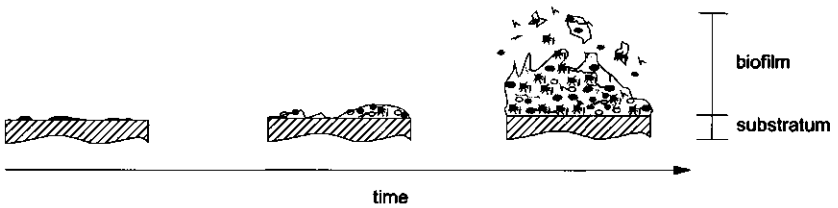


Figure 3 - Schematic presentation of the formation of a biofilm. Adapted from [21].

Most naturally developed biofilms contain different microbial groups, each with its own needs and given growth and kinetic parameters. Because of the substrate gradients prevailing in such processes, the microorganisms within the biofilm compete both for space (the closer to the substrate, the less limited they are) and for substrates. Because of these competitions, the biofilms will develop into layered structures where the cells closer to the surface are those with the highest growth rates and affinity for the respective substrates [41,42]. For detailed information on biofilm formation and their application to wastewater treatment the reader is referred to the books of Characklis and Marshall [38], Characklis and Wilderer [37], Tijhuis, [17] and Melo et al. [43] and to the reviews of Bryers & Characklis [44], Bryers [39] and van Loosdrecht & Heijnen [35, 41].

A large variety of reactor configurations based on such biofilm processes has been developed and applied on nitrogen removal systems. The most recent of these developments will be discussed later in this paper.

*Artificially immobilized cells*

An alternative way of immobilizing cells is by entrapping them in a polymeric matrix. In general, by this procedure, a cell suspension is mixed with a suspension of the entrapping (pre)polymer and the mixture is then allowed to gelify or polymerize either to their final form (generally beads of 0.5 to 3 mm) or to a form from which the biocatalyst particles can be later shaped (e.g., cubes or cylinders cut off of polymer sheets or fibers). There are several principles of gel formation that include ionotropic gelation, thermal gelation, cross-linking and polymerization, among others. The materials used can be either natural polysaccharides (such as alginate, carrageenan, agar, chitosan) or synthetic polymers (polyvinyl alcohol, polyacrylamide, polyurethane and its derivatives). Extensive overviews on both the techniques and materials used for artificial cell immobilization can be found on the book of [45] and the reviews of Willaert and Baron [20], Scott [46], and Klein and Vorlop [47].

One of the most common methods used for the study of nitrogen removal processes with artificially immobilized is that in which nitrifiers, denitrifiers or mixtures of both are entrapped in alginate or carrageenan beads formed through dropwise extrusion of the gel suspension into a solution containing their counterions (see Wijffels & Tramper [21] and references therein, see Figure 4).

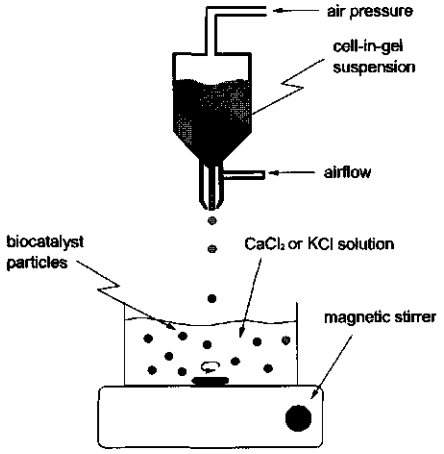


Figure 4 - Schematic representation of dropwise extrusion of alginate or carrageenan in a $CaCl_2$ or KCl solution where gelation is initiated. Adapted from reference [48].

A main difference of such a process compared to those of natural attachment, is that initially the cell populations are well defined, quantified and uniformly distributed throughout the biocatalyst particle. Due to the mass-transfer limitation processes above described, biomass and solute gradients develop across the beads, resulting, likewise the biofilm processes, on a layered structure of biomass and steep substrate and product profiles.

These aspects as well as the application of artificially immobilized cells to nitrogen removal will be discussed later in this paper. Both fundamental and applied aspects of natural and artificially immobilized cells and their relevance for nitrogen removal processes are comparatively discussed in the section *Biofilms versus artificially immobilized cells* below, after having described treatment systems based on both processes.

**Conventional Biofilm Processes**

Since biofilms develop naturally onto solid surfaces, technologies that rely on naturally-immobilized biomass were the first to develop as an alternative to activated-sludge processes in wastewater treatment. These developments set forth during the last century so that at the present numerous processes using naturally attached cells have been designed and built up.

*Trickling filters*

The trickling filter is the most widely used immobilized-cell system. It is a percolating filter consisting of a bed packed with porous supports on which a biofilm develops (Figure 5). These filters are also known as oxidation beds or dry biofilters. In the past, the support materials used were relatively large pieces of lava or stone, which have relatively limited surface areas (50-100 $m^2m^{-3}$). Currently, low density plastic supports (e.g., polystyrene or polyvinyl chloride) with specific surface areas up to 300 $m^2m^{-3}$ are used [49-51]. The wastewater containing the nitrogen compounds is distributed uniformly at the top of the bed and percolates through the supports. Nitrifying conditions are provided by supplying air at the bottom of the bed.

Ammonium removal rates up to 2 g-N. $m^{-2}.d^{-1}$ (0.6 kg-N.$m^{-3}.d^{-1}$) were reported in such trickling filters [53,54]. Further conversion of nitrite and nitrate is normally done by coupling the effluent of the trickling filter to an activated-sludge tank or eventually to another "trickling filter" (which is then referred as a packed-bed or anaerobic filter [55] although this last configuration is less usual. Denitrification can be in achieved in such filters by restraining the availability of oxygen [56-58]. Sagberg *et al.* [58] successfully

achieved coupled nitrification and denitrification in a modified two-stage fixed-film process.

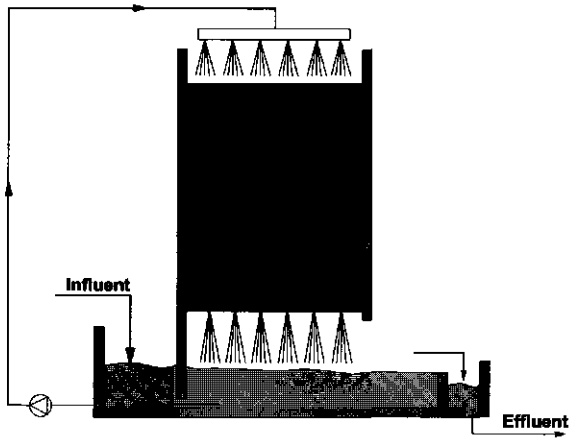


Figure 5 - Schematic representation of a trickling filter (adapted from reference [52]).

To our knowledge, however, there are no reports on integrated nitrification and denitrification in single-stage trickling filters. Compared to the activated-sludge systems, trickling-filter processes have lower operational costs, consume less energy and the reactors themselves occupy less space, but do not necessarily perform much better than activated-sludge processes (little or no improvement in the specific surface area, see Table 2) and are very liable to clogging and intense production of bad odours due to bacterial growth [49-55].

*Rotating biological contactors*

In rotating biological contactors (RBC) the biofilms are attached to disks. A large number of these disks are closely mounted on a shaft that slowly rotates (Figure 6). The disks are partially submerged (40-60%) in the wastewater, so that they contact alternately air and wastewater as the shaft rotates (typically 1 to 5 rpm). The thickness of the biofilm is self-limited by the shear tensions caused by rotation of the closely packed disks through the medium. These reactors have a specific surface area of 150 to 300 $m^2m^{-3}$ and biomass concentrations of up to 30 $kg.m^{-3}$ can be attained [52].

Nitrification and organic-carbon removal strongly depend on the oxygen dissolved in the biofilm. Due to oxygen diffusion limitation caused by nitrifying and heterotrophic activity, aerobic and anoxic zones develop within the biofilm enabling thus denitrification to occur in deeper parts of the biofilm.

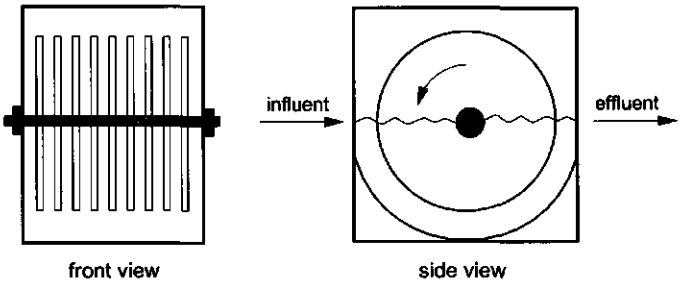


Figure 6 - Schematic representation of a rotating biological contactor (from [59]).

Watanabe and co-workers [59] reported on stratified biofilms containing heterotrophic bacteria at the surface, nitrifying microorganisms just underneath and, deeper in the biofilm, the denitrifying microorganisms. The process relied both on the occurrence of aerobic and anoxic zones within the biofilm and on the alternate exposure of the biofilm to air and the more or less anoxic liquid by rotation of disks. This makes the system relatively sensitive (and thus unstable) to changes in the environmental conditions as this demands continuous tuning of the operating conditions (such as the rotating speed and carbon loads). The RBC processes in general have relatively low operating costs and are simple to operate, but their capacity is still limited because the reactors cannot be too large due to mechanical constraints [17, 52].

*Submerged filters*

Similarly to trickling filters, submerged biofilters are reactors packed with carrier materials to which biofilms attach. In these reactors, however, the bed is continuously submerged in the wastewater (Figure 7). Several types of packing materials can be used such as expanded clay, pumice stone, polystyrene, sand, ceramics or coal [52,60]. In such systems, the bed has normally a height of 2 to 4 m and a specific surface area that can vary largely between 200 and 1000 $m^2.m^{-3}$, depending on the type, size and form of the carriers used (e.g., small polystyrene rings or beads of sand or expanded clay [55, 60-64]. In submerged biofilters, the wastewater streams can be supplied either up- or downwards (Figures 7a and 7b, respectively). Both types of reactors can be operated either aerobically or anoxically by supplying or not air at the bottom, respectively. In the last decade, these kind of reactors have become increasingly popular and are available commercially.

The most common downflow biofilters are the Biocarbone filters (Denitropor for anoxic configurations) supplied by the French OTV and Danish Krüger [51,60,62], whereas the most used upflow reactors are those of the French Degremont under the name Biofor

[18, 63-65] (Nitrazur for anoxic configurations [66]). The nitrogen removal capacities of both types of systems are similar. Integration of nitrification and denitrification with these biofilter systems is commonly done by coupling two submerged filters in series and applying some form of recirculation (normally an anoxic downflow filter for denitrification followed by an upflow aerated filter for nitrification [51,67,68]). Often, an external carbon-source has to be supplied for effective denitrification. Nitrification rates ranging from 0.4 and 1.5 kg-N.m$^{-3}$.d$^{-1}$ and denitrification rates between 0.5 and 3 kg-N.m$^{-3}$.d$^{-1}$ (global nitrogen-removal efficiencies of 80-90%) in sequenced biofilters have been reported for temperatures between 10 and 25 °C [42,58,64,69,70].

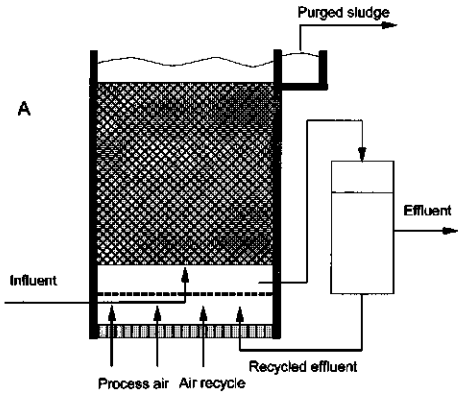


Figure 7 - Submerged filters: A) upflow; B) downflow. Adapted from Garrido [52].

The so-called second generation of submerged filters consist of biofilters in which the support material is less dense than the medium (e.g., polystyrene, polypropylene or polyethylene beads). These filters are called floating bed reactors and are also available commercially (Biostyr from the French OTV, Biobead from the British Anglian Water Services and Denipor from the German Preussag, see also Lazarova & Manem [18]). Several studies report somewhat better nitrogen-removal performances with such reactors than with those of fixed bed biofilters [71-73]. Also, since in these reactors the support materials float, less energy is necessary (and thus the operational costs are lower) for pumping the medium through the filters. Another advantage is that backwashing is facilitated [71]. Recently, a new biofilter reactor concept (Bioregenerator) with reduced backwash requirements has been presented [72]. This biofilter is operated as a floating, downflow filter with aeration at the bottom. The carrier particles (buoyant, synthetic spheres with deep grooves) are continuously removed at the top and passed through a mechanical sludge separator that removes the excess biofilm. The cleaned carriers are then re-introduced at the bottom of the reactor.

This reactor type has already been tested successfully at full-scale for nitrification, although with removal rates (0.6 kg-N.$m^{-3}$.$d^{-1}$) lower than in other biofilter systems. This was basically due to the relatively low reported specific surface areas of the carriers (240 $m^2$.$m^{-3}$). So far, no integrated nitrogen removal with such a reactor concept has been reported. In general, biofilter systems consume more energy than those based on activated-sludge, and both the operational and installation costs are higher. They are, however, much more compact than suspended reactors (also because of the possibility of construction in the height) and can handle higher loads. An indicative comparison between these and both other compact systems and activated-sludge reactors is shown in Table 2.

*Moving beds*

A process developed to reduce the problem of increasing pressure drops in biofilters and thus frequent backwashing is the moving-bed reactor. These are reactors in which the biomass attaches onto porous carriers that are kept moving by mechanical agitation and/or aeration (see Figure 8). The carriers are commonly made of polyurethane-foam rings [74] or cubes [75,76], or of polyethylene cylinders [77]. These synthetic foams potentially provide a specific surface area up to 1000 $m^2$.$m^{-3}$, although only 20-40% is actually used due to clogging of the pores by growth of biomass[18,77]. Nitrification and denitrification rates of about 0.3-0.6 and 0.8-1.2 kg-N.$m^{-3}$.$d^{-1}$, respectively, have been reported both at laboratory [74,78] and full scale [79]. Integration of nitrification and denitrification has been achieved both at lab and full scale either by placing moving beds in aerobic/anoxic subsequent compartments [27,74,80] or reactors [77] or by applying aerobic/anoxic cycles to a set of reactors (MBSBBR process, [77]).

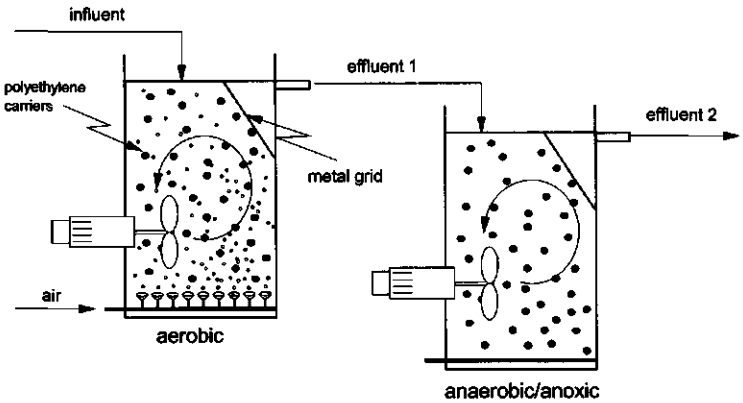


Figure 8 - Moving bed reactors. Denitrification follows nitrification. From [80].

Table 2 - Overview of the main process units used in nitrogen removal.

| Basic process units (applications at industrial scale if applicable) | Carrier type | Carrier diameter (mm) | Filling rate (% v/v) | Specific surface ($m^2.m^{-3}$) (before biofilm formation) | Biomass concentration ($kg.m^{-3}$) | N-removal capacity ($kg.N.m^{-3}_{reactor}.d^{-1}$) | Sludge production ($kgDM.kgN^{-1}$) | Energy requirements ($kWh.m^{-3}_{treated\ water}$) | Space utilization ($m^3.kg\ N\text{-}removed^{-1}.d^{-1}$) | Representative references |
|---|---|---|---|---|---|---|---|---|---|---|
| Activated-sludge | - | - | | 50-150$^{flocs}$ | 0.5-6 | 0.2-0.6 N+D | 0.4-1 | 0.05-0.2 | 1-2 | [49], [60], [91], [171], [90-93] |
| Trickling filter | polystyrene, expanded clay | 2-5 | 40-60 | 150-300 | 5-15 | 0.4-0.8 N | 0.3-1 | 0.1-0.3 | 0.5-0.8 | [50], [51], [53-58] |
| Rotating Biological Contactor | * poly-methyl-metacrylate, polysterene | - | 40-60 | 200-400 | 5-15 | 0.2-0.5 N+D | 0.3-1 | 0.1-1 | 0.5-0.7 | [59],[60],[194], [195],[238], [318] |
| Upflow filters (Biofor, Nitrazur, Oxyazur, Denitropur) | expanded clay, pumice stone, sand, ceramics | 1-5 | 40-60 | 200-700 | 5-15 | 0.4 - 1.4 N<br>0.8-3 D | 0.3-1 | 0.02-0.2 | 0.45 - 1 | [42], [63],[64], [66-70] |
| Downflow filters (Biocarbone, Denitropor) | expanded clay, pumice stone, sand, ceramics | 1-5 | 40-60 | 200-700 | 3-10 | 0.2 - 1.2 N | 0.3-1 | 0.02-0.1 | 0.6 - 0.1.2 | [51],[60-64]], [142] |
| Floating bed (Biostyr, Biobead, Denipor) | polystyrene, polypropylene, polyethylene | 1-5 | 30-50 | 300-700 | 5-15 | 0.5-1.5 N<br>1.2-3 D | 0.3-1 | 0.005-0.1 | 0.3-0.8 | [18],[71-73] |
| Moving beds (KMT) | polystyrene, polyurethane | 2-10 | 30-50 | 100-500 | 3-10 | 0.3-0.6 N<br>0.8-1.2 D | 0.3-1 | 0.03-0.15 | 0.4-1 | [18],[27], [74,-81] |
| Fluidized beds (Oxitron, CIRCOX) | sand, clay, carbon | 0.2-2 | 20-40 | 1500-3000 | 10-60 | 1-3 N<br>2-6 D | 0.05-0.20 | 0.09-3 | 0.1 - 0.30 | [15],[16],[18], [28],[34], [95-123] |
| Gas-lift reactors (PEGASUS, CIRCOX, Gist-Brocades ) | sand, basalt, polymeric beads | 0.2-2.5 | 5-20 | 1500-3000 | 10-60 | 1-6 N<br>3-10 D | 0.05-0.20 | 0.1-3 | 0.1 - 0.15 | [[10],[16],[17], [18],[21],[26], [28],[34],[35], [124-152],[159] |
| Membrane reactors | ceramics, polyethylene, polymeric gels | - | - | 200-1500 | 10-40 | 0.5-3 N+D | 0.05-0.20 | 3-5 | NA | [94],[95],[205], [227] |

* - disks; NA - not available
N - nitrification; D - denitrification

This table was made on the basis of the references mentioned. Temperatures varied between 17 and 30 degrees, which makes direct comparison more difficult. The figures in the table should be thus considered as indicative trends only.

In average, the total-nitrogen removal rates achieved in these systems were in the order of 0.4-0.6 kg-N.$m^{-3}$.$d^{-1}$ (see Table 2). According to Lazarova & Manem [18], the main problem associated with these processes is to keep the supports moving and to prevent their flotation. Stirring cannot be too vigorous as it may lead to damage of the carriers and respective biofilm. This in turn also leads to a decrease in the mass transfer coefficients and thus a reduction of the maximum attainable treatment capacity of the system. An obvious advantage in comparison to other fixed-film reactors is that no backwashing is necessary.

More detailed descriptions of biofilm reactors and biofilm processes can be found on the proceedings of specialized conferences [81,85]. An overview of the most important process units used in nitrogen removal is presented on Table 2. Since almost each of the systems described were operated at different conditions, exact comparisons are not possible. For instance, the nitrogen conversion rates reported refer to temperatures that ranged from 17 to 30°C. The dependence of these rates with the temperature is given for nitrifying cells in Figure 4 above. Hence, the figures in the table should be thus considered only as general trends. For more thorough discussions see also the reviews of [18,55,69,86,87] and the evaluation studies of Kruit & Wiegant[62] or STOWA[60]. For more detailed descriptions of conventional activated-sludge (suspended-cell) systems the reader is referred to the works of [49,60,88-94].

**New Generation Bioreactors**

Many of the conventional systems based on immobilized biomass described above have led to considerable improvements in the effectiveness and efficiency of nitrogen-removal processes. Several of these systems are being widely used in wastewater treatment, either as a result of the upgrading of existing processes or of the build-up of new treatment units. Since relatively much volume of biomass accumulates in such reactors and the flow is in most of them of the type semi-plug, these configurations are often appropriate for the treatment of large flows of low-concentration waste or groundwaters. However, for waste streams with higher concentration of nitrogen compounds, these reactor types are not compact enough and still have limited overall volumetric productivities [35]. Basically this is due to a) an as yet low biofilm specific-surface area (200-500 $m^2$.$m^{-3}$, see Table 2); and b) poor mass transfer parameters as a consequence of deficient mixing. Moreover, and with exception of the moving beds, the processes above described have in common the important drawback of the need of frequent backwashing due to clogging of the beds. And still, despite of some progresses regarding compactness, in most of the systems described nitrification and denitrification

are as yet carried out in separate reactors with some form of recirculation between them. These issues demanded thus further developments in the nitrogen-removal technology.

***Fluidized beds***

The most rational way of increasing the contact surface of immobilized biomass with the surrounding medium is by reducing the size of the immobilization supports (the positive effect of an increase in porosity of the carriers is rapidly neutralized by clogging of the pores). However, for a given density, the size of the particle should not be too small as it would make difficult the separation of carriers from the effluent. In practice, specific surface areas up to 3000-5000 $m^2.m^{-3}$ are attained by using particles of e.g., sand, basalt, pumice stone or expanded clay with diameters between 0.5 and 2 mm [15,16,18,95,96]. Yet, the total effective specific surface area available tend to be lower than these theoretical values because the degree of filling rarely goes over 30-40% [18]. Indeed, instead of packing the particles in a tank or bed such as done in submerged filters, these supports can be kept in suspension in a tall column by the pumping through it the wastewater at high velocities. These velocities are attained by recirculating continuously the wastewater through the bed at such rate that particles fluidize. The oxygen necessary for nitrification is normally provided by aeration of the recirculating medium outside the reactor itself (Figure 9).

The overall removal productivities attained in these reactors are in general much higher than those in conventional fixed-bed system. This is due both to the reduced size of the carriers (larger specific surface for mass transfer and higher biomass concentration) and due to the low hydraulic retention times. By increasing the concentration of biomass and thus their "age" in the reactors (lower growth rates), less surplus sludge is produced [16,35]. Furthermore, by fluidizing the biofilm particles, no clogging occurs and thus no backwashing is necessary. Yet another advantage is that these, (generally tall and narrow) reactors can be easily closed to avoid odour emissions and noise.

Fluidized beds designed for nitrification (aerobic) and denitrification (anoxic) processes are available commercially (Oxitron by Dorr-Oliver, Rex by Ecolotrol/Envirex [18], and have been applied at full-scale in wastewater treatment plants.

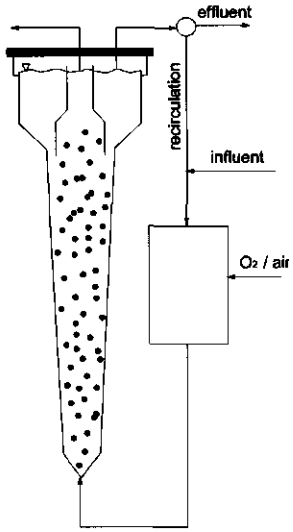


Figure 9 - Fluidized bed with aerator.

Removal rates up-to 1-2 kg-N.m$^{-3}$.d$^{-1}$ for nitrification and 2-4 kg-N.m$^{-3}$.d$^{-1}$ for denitrification have been reported in these systems [60,97-106]. Coupling of nitrification and denitrification using fluidized bed technology involves in most cases the use of reactors in series, that can be either two fluidized beds [102,107] or a fluidized bed in series with some kind of submerged filter [60,87]. Fluidized beds are also used for denitrification in upgrading activated-sludge systems for nitrification and organic-carbon removal [103, 108-110].

Despite the advantages of this technology, its application for nitrogen removal at full-scale is, with exception of the examples mentioned above, still very limited. Indeed, most of the advances related to fluidized beds in nitrification [110-114] denitrification [114-118] or both [107,119,120] are still at lab and pilot scale. The reasons for that are in general associated with scaling-up problems due to the difficulties in controlling the bed expansion and biofilm thicknesses, influent distribution devices and/or oxygen saturation systems [18,. 51, 94,99, 121-123].

***Gas-lift reactors***

A main limitation of aerobic fluidized beds is that the transfer of oxygen from the gas to the liquid phase is relatively slow and therefore oxygen depletion along the length of the reactor may occur if the oxygen demand is high, as in the treatment of high-strength wastewaters [17,21]. Ammonia removal from such wastestreams requires, in general, a

large increase of the recirculation rates or/and oxygenation with pure oxygen, which increases the operation costs. Furthermore, there are important hydraulic limitations at too high recycling rates. As an alternative, the reactor might be aerated directly by sparging air at the bottom, creating thereby a three-phase (bubble column) reactor. The operation of such reactors is, however, frequently hampered by coalescence of bubbles and flow inhomogeinities [18]. The hydrodynamic performances (mixing, circulation, fluid velocity) of these reactors can be largely improved by inserting a draught tube in the column [15, 124-126].

Circulation of the fluid and biocatalyst particles is created by the density difference between the aerated and non aerated sections of the reactor (see Figure 10).These reactors are commonly called (internal) air-lift loop rectors. Another common configuration is that of the external air-lift reactors in which the aerated and non-aerated sections are connected to each other by a top and bottom horizontal section (Figure 11).

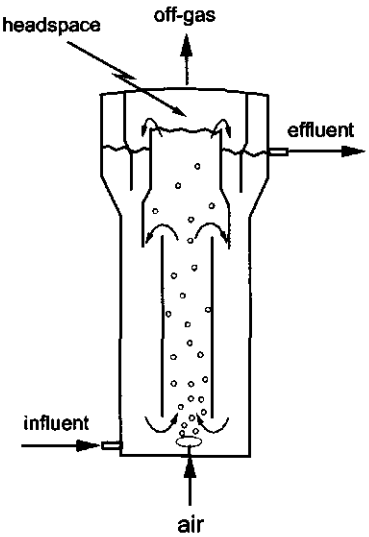


Figure 10 - Internal airlift loop reactor. Adapted from van Loosdrecht & Heijnen [35].

The support particles are retained in the reactors by some form of three-phase separators which include small secondary air-lifts at the top [15,16] or a simple grid at the reactor outlet.

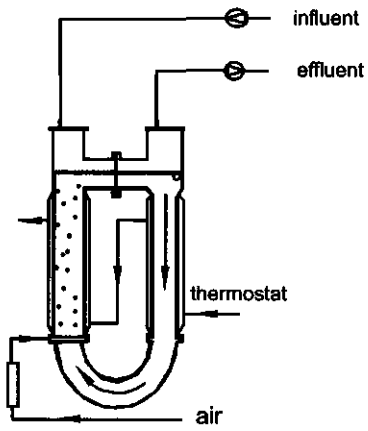


Figure 11 - External airlift loop reactor. Adapted from Wijffels et al. [135].

In the last decade extensive research has been done on the application of air-lift loop reactors for nitrification with both biofilms grown on carriers [16,17,96,127-129] and cells artificially immobilized in polymeric supports [23,25,26,33,130-142]. These studies have shown that nitrification rates up to a maximum of 6 kg-N. $m^{-3}.d^{-1}$ can be reached with biofilms (particles size between 0.2 and 0.4 mm, filling rate of 15% (v/v) at a temperature of 30°C and superficial gas velocity of 2 $cm.s^{-1}$[17,143]. This is about 10-15 times higher than those commonly achieved in activated-sludge systems (see Table 2). In steady-state, these conversion capacities are mainly limited by the transport of oxygen from the gas phase to the biofilm surface [143]. In most situations, however, these productivities are actually more than enough to achieve effective nitrification. In the systems based on entrapped cells, the maximum capacities reported so far are about half of those based on biofilms, which is likely due to the relatively large particles used (2-3 mm).

*Industrial scale*

Although most research with both natural or artificially-immobilized cells has been carried out at laboratory or pilot scale there are some reports on full scale. The first full-scale process (750 $m^3$) with artificially-immobilized nitrifying cells has been recently presented (PEGASUS® from Hitachi, Japan [140,144]. In this process, polyethylene glycol pellets containing nitrifying activated sludge are used for nitrification of domestic wastewater (see more details and Figures 15, 16 in the next section). The system has been operationally stable for five years and at the moment there are other units being built up Japan.

Also nitrification by naturally attached biomass in air-lift reactors has been described at full scale. Heijnen and co-workers [15,16] reported well-nitrifying 300 $m^3$ air-lift reactors from the treatment of concentrated acidified wastewaters in a yeast-production plant (Gist-Brocades, The Netherlands [1,15,16]). These reactors make part of a rather compact treatment unit that, besides nitrification, includes denitrification, methanogenesis and removal of sulfur compounds (see Figure 12).

The systems avoids the problems associated with nitrifying bacteria in a high BOD effluent (see discussion on the subsections *MAL* and *Bioreactors in series*) by placing nitrification last in the sequence and then by recirculating the nitrate generated in the denitrifying reactor. The anaerobic reactors are fluidized beds whereas those of nitrification are three-phase airlift reactors.

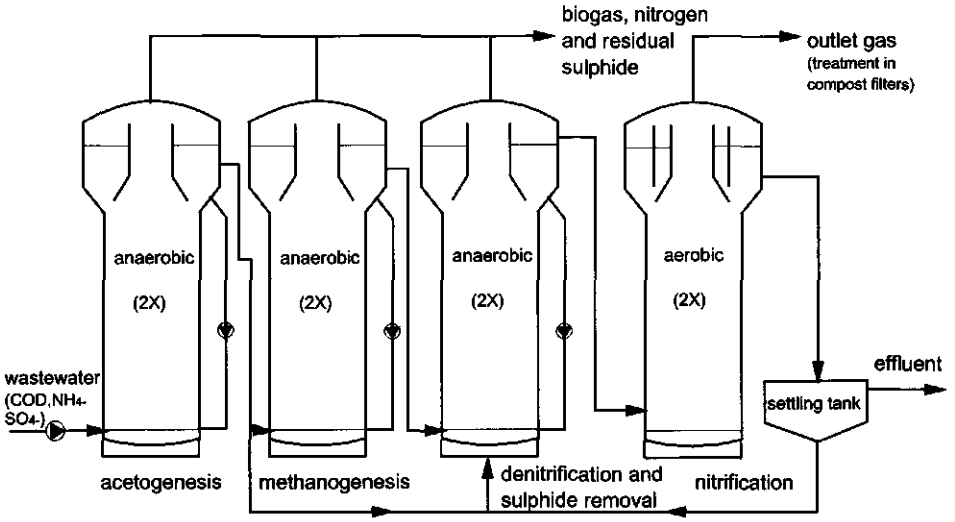


Figure 12- Simplified presentation of the wastewater treatment unit at Gist Brocades, The Netherlands. Adapted from references [1], [15] and [34].

Despite the undoubted advantages of gas-lift loop reactors (see Table 2), and except for the two plants just described, their widespread use at industrial scale is still very limited. Besides being a relatively new reactor concept in wastewater treatment (10-15 years) of which many aspects are not well known yet, such reactors are also sophisticated to construct and relatively complex to operate and scale-up [15,145]. Nevertheless, at least two independent technical-economic feasibility studies for the application of air-lift reactors with naturally attached biomass (Witteveen+Bos [146], Kampf et al. [147], both as described by Tijhuis [17]) and two specific for nitrification with artificially

immobilized cells (Chudoba *et al.*, [26], Witteveen+Bos [148]) have shown that such reactors are clearly promising (within the given scenarios) for purification of municipal wastewaters and in particular if space (and/or land price) is a problem. The savings in reactor volume for a given needed capacity compared to nitrification in a conventional activated-sludge system are considerable if the immobilization support remains stable for at least two months [24,149].

***Compact configurations based on fluidized-bed and gas-lift-reactors***

*Bioreactors in series*

Since the functioning of a gas-lift reactors relies on the injection of a gas at the bottom of a given section of the reactor, virtually all the applications with these reactors use air as sparging gas. This implies that in general air(gas)-lifts are only used for nitrification, with a few exceptions that will be discussed below ([150], Martins dos Santos *et al.*, unpublished results). For complete nitrogen removal (i.e. with denitrification), these reactors are normally coupled in series with fluidized beds [103,15], submerged filters or conventional activated-sludge tanks[151,152] with some form of recirculation between them. For instance, van Bethum and co-workers [152] have designed a system involving an air-lift reactor with attached nitrifying biomass, coupled in series (in a pre-denitrification configuration) with an anoxic agitated reactor containing basically suspended denitrifying biomass. An innovative aspect in this nevertheless relatively common configuration, is the manipulation of the hydraulic retention time in the system so as to obtain "hybrid biomass" [152]. This concept of "hybrid biomass" means basically that both the nitrifying and denitrifying populations co-exist in the reactor system, with the slow-growing nitrifiers attached onto the carriers whereas the fast-growing denitrifying biomass remains primarily in suspension. Such distribution is achieved by applying a retention time that is shorter than the reciprocal maximum growth rate of the nitrifiers but longer than that of the denitrifying population. This is based on the observation that biofilms develop only under conditions where suspended cells are quickly washed-out [34,36,96,143]. In preliminary experiments, the authors (van Benthum, personal communication, 1996) obtained about 75-80% nitrogen removal for an ammonium load of 5 kg-N.m$^{-3}$.d$^{-1}$ in a 3 liter air-lift reactor coupled with a 2.4 liter chemostat, hydraulic retention time of 10 hours and a recirculation ratio of about 6.5. The external C- source was supplied in the anoxic chemostat. From their design studies, such a system seems to be particularly appropriate to treat wastewater with high ammonium concentrations (typically more than 0.5-1g-N.L$^{-1}$). For dilute streams (less than 0.5 g-N.L$^{-1}$) or if very low ammonium effluent concentrations are required (less than 0.02 g-N. L$^{-1}$) the system becomes mainly limited by the maximum

(recirculation) flow allowed for a given suspended reactor volume. That means that for effective nitrogen removal an extremely large anoxic reactor would be needed, which is technically nor economically feasible. In this case, this is basically the same limitation that holds for conventional nitrifying systems with activated (suspended) sludge described above. Therefore, another configuration should be used, possibly a process involving immobilized or attached denitrifying cells [152].

Such a configuration was used by Jördening et al. [103] who reported on nitrogen removal rates up to 3.6 kg-N.$m^{-3}$.$d^{-1}$ with 90 % efficiency using an anoxic expanded bed (7 l) containing 75% (w/v) porabact® granules (denitrification with attached biomass) in series with a 20 L air-lift filled with 24% (w/v) sand carriers (biofilm nitrification). The hydraulic retention times were 3-4 hours and the recirculation between the nitrifying reactor and the denitrifying bed ranged from 10 to 55 L.$h^{-1}$.

***Compact units***

As shown in the previous sections and by the last examples, reactor systems based on fluidized beds and air-lift reactors are quite compact and require much less volume and space (due to their constructions in height) than activated-sludge systems. Nevertheless, the nitrification and denitrification processes, similarly to the conventional configurations in activated sludge systems, are still carried out in separate reactors with some form of recirculation between them. Therefore and since in general it is more expensive to have two reactors instead of one, quite a lot of research has been done to find ways of combining nitrification and denitrification in the same reactor or treatment unit while maintaining the advantages of fluidized bed or air-lift-based systems. Several of the compact systems developed as result of these research efforts are described below.

*Alternate aerobic/anoxic conditions*

The possibilities of using a single fluidized bed or air-lift loop reactor (or a modified version thereof) has been investigated by several groups. For instance Matsui and co-workers [153], have reported on a modified internal air-lift reactor that was intermittently aerated (cycles of 15 minutes) with continuous inflow of wastewater (retention time of 2.9 hours). By this mode of operation, the reactor behaved as an air-lift during aeration and as a fluidized-bed during "anaeration". Nitrification and denitrification took place in these two periods, respectively. The reactor was filled (up to 8% of the reacting volume) with small (0.2-0.3 mm) sand particles onto which a biofilm developed. The nitrogen removal efficiency reported was about 90-95% for (total)nitrogen concentrations of about 30-40 g.$m^3$ at 20°C.

Van Benthum and colleagues [152] have worked out a similar reactor concept for a three-phase air-lift reactor filled with small basalt particles (0.2-0.3 mm) covered by attached biomass. In their process, however, the reactor is continuously gassed so that it behaves as a gas-lift reactor at all times. Initially, fresh air is sparged in the reactor providing aerobic conditions for nitrification. After a given time, supply of fresh air stops and the off gas is recirculated through the reactor for another period of time. During recirculation, the oxygen remaining in the gas phase is rapidly depleted and the reactor becomes anoxic. Within this period, either fresh wastewater containing COD or an external C-source is added so that denitrification may take place optimally. The operation of the reactor is carefully controlled ensuring that no C-source is left during the aerobic period. This prevents the COD to be consumed aerobically reducing thereby both the nitrification and denitrification capacities. The aerobic period comprises both the sparging with fresh air and the time during which oxygen has not yet been depleted from the gas phase.

In preliminary studies, the authors refer to about 80% removal efficiency for a nitrogen load of 2.5 kg-N.$m^{-3}$.$d^{-1}$ in a 3 liter reactor (van Benthum, personal communication, 1996). The hydraulic retention times varied between 5 and 10 hours whereas the aeration cycles varied between 30 minutes aerobic / 30 anoxic, and 24 minutes aerobic / 11 anoxic. According to their design studies, the oxygen depletion time is one of the most important factors that ultimately determines the total capacity of such a reactor system. Similarly to their two-vessel configuration scheme, this system is based on a "hybrid concept" [152] in which the nitrifying biomass is primarily attached whereas denitrifiers are mainly in suspension. Thus, similarly to the two-vessel system and for the same reasons, this configuration is suitable mostly for the treatment of high to medium concentrated wastewaters, whereas for effective removal at lower ammonium concentrations, growth of denitrifiers as immobilized biomass is necessary. The system is currently under further optimization.

*Simultaneously aerobic/anaerobic fluidized bed*

In a different approach, Fernandez-Polanco and co-workers [119-120] described an anaerobic/aerobic pilot-scale fluidized bed for the simultaneous removal of nitrogen and organic carbon (see Figure 13). Basically, the reactor is a tall fluidized bed in which air is sparged halfway the column. Therewith, the reactor is divided in two parts: a lower anoxic zone where denitrification and anaerobic organic oxidation occur, and an upper aerobic zone where nitrification and aerobic oxidation of carbon compounds takes place.

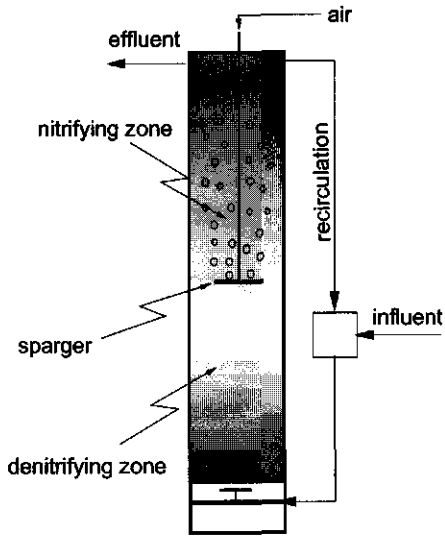


Figure 13 - Fluidized bed with anoxic and aerobic zones as proposed by Polanco et al. [119].

Fluidization of the anoxic zone is done by recirculation (pumping) of part of the reactor effluent, after going through a gas-liquid-solid separator. By placing spargers at different heights in the reactor, the volumes of the aerobic and anoxic areas are adjusted as needed. The support material consists of 0.3-0.4 mm pulverized pumice-stone. The separation of the reactor in two zones has led to a certain degree of segregation of the microbial populations involved, which has been experimentally determined by activity assays [119]. Although the nitrogen removal rates reported are still modest (0.2 kg-N. $m^{-3}.d^{-1}$ together with 2 kg-COD. $m^{-3}.d^{-1}$), this compact, single-staged construction seems promising for wastewater treatment. This group in presently optimizing the process to increase the treatment capacity. A comparable system was developed by Holló and colleagues [154] who used a single fluidized bed for integrated nitrification and denitrification. In their process, the ammonia-containing stream saturated with air or pure oxygen is supplied at the base of the column. In the first half to three-quarters of the column nitrification takes place with concomitant consumption of oxygen. In the second part of the column, where of oxygen is depleted organic matter is supplied so that denitrification can occur. The advantages and drawbacks of this system are similar to those of the Polanco´s process.

*Recirculation by air-lift effect*

Hano *et al.* [129] have reported simultaneous nitrification and denitrification in a single lab-scale air-lift reactor having also concomitantly aerobic and anoxic zones. In the reactor, the riser functioned as aerobic nitrifying zone whereas in the downcomer anoxic denitrifying conditions prevailed. This was achieved by carefully manipulating the air-flow rates and thus the recirculation velocities between riser and downcomer. The wastewater was first introduced in the external annulus (downcomer, see Figure 14) and then flowed into a draught tube accompanying the sludge flocs by the air-lift action. In this way, the organic carbon present in the wastewater is mostly used as an electron donor for denitrification and only little carbon is left for aerobic oxidation. Nitrification in the riser could then proceed with little interference of heterotrophic growth.

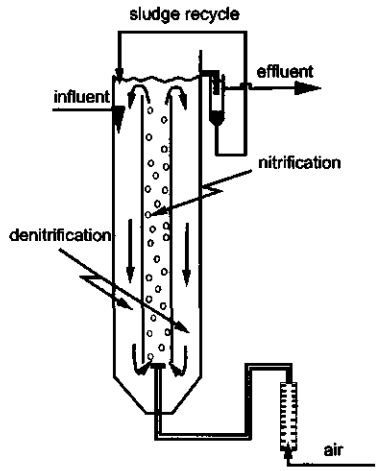


Figure 14 - Nitrogen removal in an airlift reactor as proposed by Hano et al. [129].

The extent of both nitrification and denitrification depended strongly on the ratio between the riser and downcomer, gas flow-rate and retention time. Since the nitrification and denitrification processes have opposing requirements, there were optimum values for each of these parameters (maintaining the other constant). For instance, an increase in the airflow rate enhanced nitrification and thus the overall removal efficiency. However, this also promoted the circulation rate between riser and downcomer and thus, beyond a certain level, aerobic conditions prevailed in the downcomer, resulting in a reduced denitrifying activity and increased aerobic organic oxidation. The authors were able to simulate satisfactorily these effects by developing a mathematical model based on kinetic equations for consumption of substrates within the

flocs and considering the liquid flow in both riser and downcomer as plug flow. Besides the insight gained in the processes involved, the use of a model greatly facilitates the design of such a reactor system. Nitrogen removal efficiencies of a maximum of 90% were achieved for total-N influent concentrations of $20g.m^{-3}$ at 20°C and at retention times of 6 hours in a 11.5 $dm^3$ reactor. The main limitation of this system is the relatively short residence time (few minutes) in each zone during the circulation of liquid. Because of that, a high recycle ratio is needed and thus the plug-type-flow of both sections tend to shift to a completely mixed flow which leads to incomplete nitrogen removal. Since the liquid residence and mixing times tend to increase with the scale [155,156]), the authors expected better performances at industrial size. Therefore, they proposed the use of deep-shaft reactors for the removal of nitrogen compounds from wastewaters.

*Deep-shaft*

Deep-shaft systems consist of large (30 to 300 m in height) air-lift reactors "plugged" in the ground (see Eggers et al [157] and references therein ). Such reactors with activated-sludge have been used successfully at industrial scale (the large majority in Japan and USA) for carbon removal of domestic wastewater. There are however no reports on the use of such reactors for nitrogen removal. The major reason for that seems to be the insufficient sludge age in the reactors (retention times of one to two days) for effective removal of nitrogen compounds [157]. This is also basically the main limitation in activated-sludge systems in general. It is thus conceivable that such problem could be solved using immobilized-cell technology. In theory, a deep-shaft-like system combined with the use of immobilized cells (either entrapped artificially or naturally attached) could be appropriate for effective and efficient removal of nitrogen from wastewaters in densely-populated regions with very severe limitations of space. Thorough technical-economical feasibility studies would be necessary to evaluate this possibility.

*PEGASUS®*

The company Hitachi, in cooperation with the Japan Sewage Works Agency have used the principle of recirculation by an air-lift effect to develop a compact nitrogen removal system using nitrifying sludge entrapped in pellets of polyethylene glycol (PEG) [158-160]. The bioreactor comprises a denitrification tank with activated sludge followed by a nitrifying compartment that is filled (up to 7-8%) with small PEG pellets (Figure 15). Air is sparged through disk air diffusers uniformly installed at the bottom of the nitrifying tank. Part of the outlet is discharged into a final settling tank, while the remaining portion is recycled to the denitrification tank due to the density difference

between the bulk of both compartments (air-lift effect). Both the outlet of the nitrification tank and the recirculation channel are equipped with a wedge-wire screen to prevent the washout of the pellets. Such a reactor system ("PEGASUS®"[159]) has been operated successfully at a 750 $m^3$ scale for about 6 years in a municipal wastewater treatment plant.

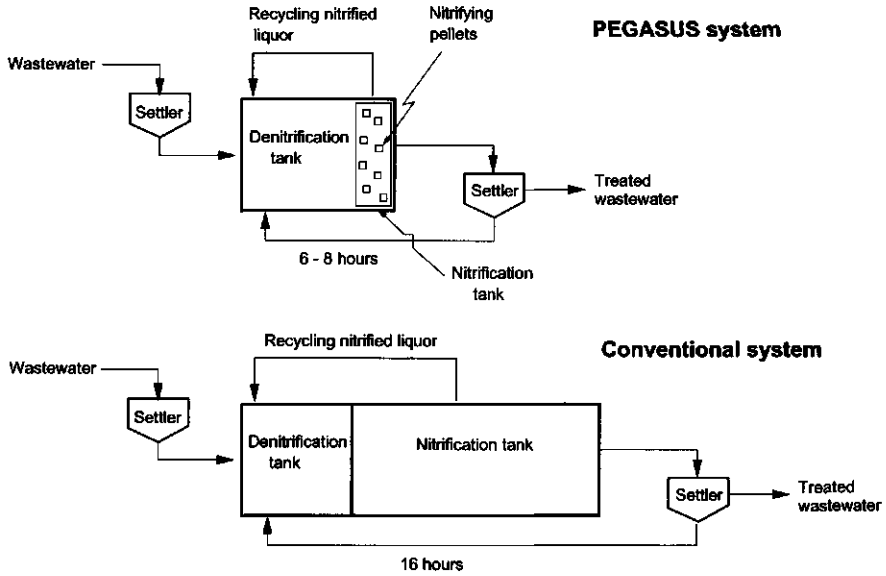


Figure 15 - The PEGASUS process according to Emori et al [140]. The pellets present in the nitrifying tank are polyethylene glycol cubes with entrapped nitrifying sludge.

The Pegasus bioreactor was constructed by adapting one section of an existing conventional activated-sludge facility and has a maximum capacity treatment of 3000 $m^3.day^{-1}$.(Figure 16). The nitrogen removal rates reported were about four-fold higher than those of conventional activated-sludge systems for temperatures between 15 and 30 °C and even 8-fold higher for temperatures between 8 and 14 °C. The total retention times were about 8 hours of which 4.8 were needed for the denitrification tank and 3.2 for the nitrification tank. The nitrified liquor was recirculated at a fixed ratio of 300%. These retention times are about the half of those of an optimized conventional sludge process [159,160].

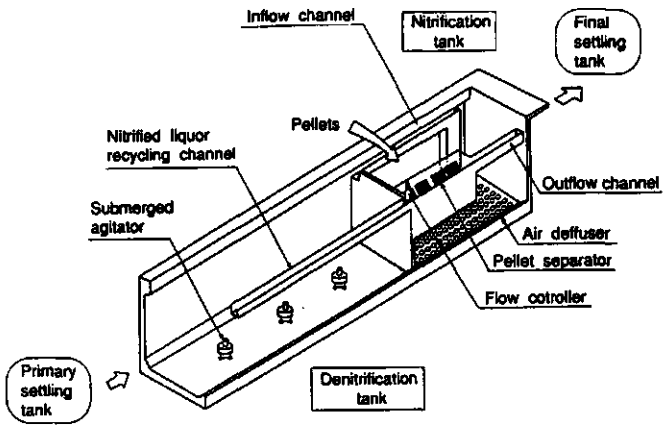


Figure 16 - The PEGASUS reactor [140]. Reproduced by permission of Elsevier Science Inc.

Also the space requirements are reduced by 50%. Possibly, the treatment capacities could be further expanded by increasing the amount of pellets in the reactor, which is in the current process relatively low (7.5%v/v).

As already mentioned above, this treatment plant is the only facility reported so far that makes use of artificially immobilized cells for environmental applications at full-scale. Several technical-economical feasibility studies in which such an immobilized system was compared to conventional activated-sludge and biofilm processes [159,26,148,149] have shown that the processes based on immobilized-cell technology are clearly attractive for effective simultaneous removal of nitrogen compounds and BOD from urban wastewaters.

*Multi-stage air-lift*

Recirculation of the nitrifying liquor in an activated sludge process was first introduced by Ludzack and Ettinger in 1962 [161] for their single-stage pre-denitrification system. Significant improvements of this technique have led to the development of relatively compact activated-sludge systems such as that of Hano and co-workers described above. Another innovative application of this concept involve the use of a multiple-stage air-lift reactor (Figure 17, [162]).

Basically, the process is identical to a multi-stage conventional activated-sludge system in which the influent wastewater is introduced in the denitrifying compartment and the effluent of the nitrifying compartment is partly recirculated to the denitrifying tank and partly to the subsequent treatment stage.

The influent sewage is step-fed to the respective stages at proper ratios. Although theoretically many stages can be applied, in practice two stages appear to be enough [162]. The main difference to a conventional multi-stage is that, in each stage, recirculation between paired nitrification and denitrification compartments is made by the use of an air-lift effect. The nitrification section is aerated at the bottom whereas the denitrification compartment is mechanically agitated. Transport between both sections is provided by openings at the top and bottom. Similarly to the system of Hano [129], the recirculation velocity between the cells in each stage is governed by the airflow rate, effective depth of the cells and the size and shape of the slits.

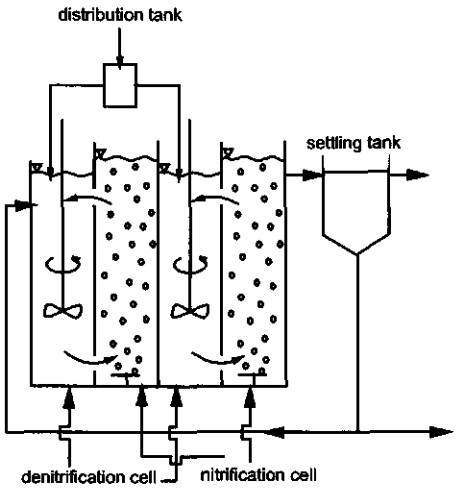


Figure 17 - The multi-stage air-lift as proposed by Taniguchi et al [162].

The authors showed that this system (using two consecutive cells) was more effective than a conventional multi(or single)-stage system and with energy savings of about 25%. Nevertheless, this multi-stage process was still based on activated sludge, by which its compactness and efficiency were still limited. Based on the considerations made above on the advantages of immobilized cell technology, it would be expected that the use of immobilized cells even for the nitrification stage alone would greatly improve its performance.

*MAL®*

The costs of series of bioreactors such as those in the process of Taniguchi above described form an important incentive to develop novel, compact (preferably single

units) bioreactor types that nevertheless incorporate the principles (advantages) of series (see De Gooijer & Tramper, [163]). Martins dos Santos and co-workers have recently proposed an integrated nitrogen removal system with a new bioreactor concept that consists of a series of concentrical gas-lift reactors with internal loop incorporated into one single vessel (Figure 18, [164,165]). Each of the succeeding gas-lifts is divided in a riser and downcomer section by a circular baffle.

This Multiple Air-Lift reactor (MAL, [166]) behaves hydrodynamically as a series of ideal mixed tanks in series (by which it can be approximated to an aerated plug-flow [167] and its usefulness has been demonstrated for a wide range of applications [168]. In the configuration proposed by Martins dos Santos et al. [165] nitrification is carried out in the first (central) air-lift by immobilized nitrifying cells and is followed by denitrification by immobilized denitrifiers in the second compartment. The effluent of the first reactor simply overflows into the downcomer of the next compartment where it is mixed with the down-flowing stream. The remaining COD is finally mineralized in the third compartment simply by suspended bacteria.

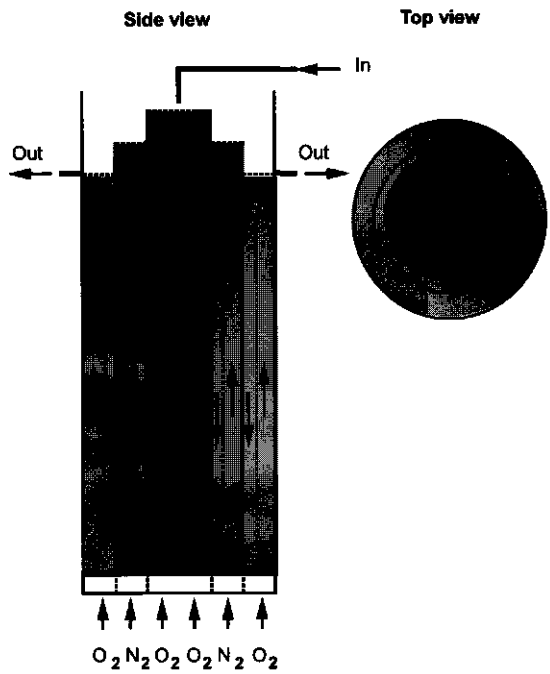


Figure 18 - Schematic presentation of the Multiple Airlift Loop reactor (MAL).

The outlets of the reactors are provided with a metal grid that prevents the beads of one compartment to flow into the other. Either part of the off-gas of the nitrifying

compartment or COD-removal compartment is used to sparge the denitrifying reactor, which can be done also simply by continuous recirculating nitrogen gas. Similarly, the COD-removal compartment can be partly sparged with the off-gas of the nitrifying reactor. Only a rather small gas flow is necessary to provide circulation and mixing in this anoxic section, which reduces the operating costs involved.

This process was studied for a wide range of operating conditions using two 3 l gas-lift reactors coupled in series, with *Nitrosomonas europaea* immobilized in κ-carrageenan beads in the first reactor and immobilized *Pseudomonas spp.* (formerly *denitrificans*) in the second. The gel beads formed about 20% of the working volume of the reactors.

If the C-source was supplied directly to the denitrifying reactor, nitrogen was effectively removed (95-99%) both for low flows of relatively concentrated wastewater (retention time 6 hours for 30-50 mM $NH^+_4$ in the influent) and high flows of diluted streams (retention time 10-15 minutes for 1-3 mM in the influent, Martins dos Santos et al., 1993, unpublished results). Both nitrification and denitrification proceeded problemless (up to an absolute maximum of 3.5 kg-N. $m^{-3}.d^{-1}$, being the nitrifying step always the limiting one) for all flow rates and N-loads applied.

*Heterotrophic attached growth*

On the contrary, supply of the acetate directly into the nitrifying reactor for short retention times (typically 2-3 hours and down to 10-15 minutes) led to a decrease in the nitrifying capacity that was closely related to the influent COD concentration. At these short retention times, heterotrophic microorganisms (typical doubling times of 0.3 to 3 hours) formed dense biofilms onto the gel beads and competed successfully for oxygen with the immobilized nitrifiers, hindering thus nitrification. Denitrification in the MAL was also affected, both at short retention times (since less nitrite was produced by nitrifiers) and long retention times for relatively low COD influent concentrations (as little COD was left from the nitrifying reactor to allow complete denitrification). This behavior follows the observation, already brought up, that biofilms develop preferably under conditions where suspended cells are quickly washed-out [34,36,96,143]. Van Benthum and colleagues [169,170], who studied the formation and disappearance of heterotrophic biofilms onto nitrifying carriers, calculated that under conditions similar to these the thin layers of attached heterotrophs may decrease the external effectiveness factor for mass transfer from 1 to 0.6 or even less.

This last aspects illustrate a common problem in wastewater treatment (i.e. hindering of nitrification by heterotrophs and/or lack of organic substrate for denitrification). This problem is commonly solved either by placing the denitrifying tank before nitrification

(and recirculating thus the effluent) or by by-passing part of the influent from the nitrifying tank to the denitrifying reactor. In both cases, however, part of the inorganic nitrogen remains to be treated. To prevent this, a common practice is to add an external carbon source (such as methanol or ethanol) to the denitrification tank and placing the nitrogen removal unit (post-denitrification configuration) after the COD removal step, which ensures then maximum nitrogen removal (see references [91,171]). An alternative is to try to find an optimum compromise where both considerable nitrification plus denitrification is attained while supplying the C-source directly in the nitrifying reactor so that neither nitrification is strongly inhibited nor denitrification is incomplete due to lack of C-source. In their study, Martins dos Santos et al. (unpublished results) varied both the influent COD concentrations and hydraulic residence time in the system and found that, for their system, such a compromise could be attained at a retention of 4 hours and a COD concentration of 250-350 $mg.l^{-1}$.

In short, the treatment process based on the MAL configuration seems appropriate for integrating nitrification and denitrification in a single reactor if the C-source is fed directly into the denitrifying section. If the C-source is supplied in the nitrifying section, its nitrogen removal capacity is limited. Its applicability is thus the greatest for treating high flows of wastewater poor in organic carbon or placed after a COD removal unit.

*BASE and CIRCOX®*

Another approach similar to that of the MAL reactor has been recently presented by van Benthum and van Loosdrecht [172]. They described what could be considered a hybrid version between their two systems summarized above (respectively, an air-lift with attached nitrifying biomass coupled to an anoxic suspension reactor, and a single airlift reactor with alternating aerobic and anoxic periods). In this hybrid system (BASE reactor), a common air-lift reactor is extended to a sort of "one-and-a-half" air-lift that includes an extra downcomer in which denitrification takes place (see Figure 19).

The nitrification and denitrification processes are, unlike those in their previous systems, both carried out by biomass attached to small (0.3 mm) basalt carriers (at concentrations up to 30 $g.l_{reactor}^{-1}$). Similarly to the MAL process, in this configuration two reactors are coupled to each other in one single vessel without the need of forced external circulation (pumping) between them. The influent wastewater is fed directly into the anoxic compartment. As in the single air-lift with alternate aerobic and anoxic periods, a critical aspect in the BASE reactor is the circulation rate between the aerobic and anoxic compartments.

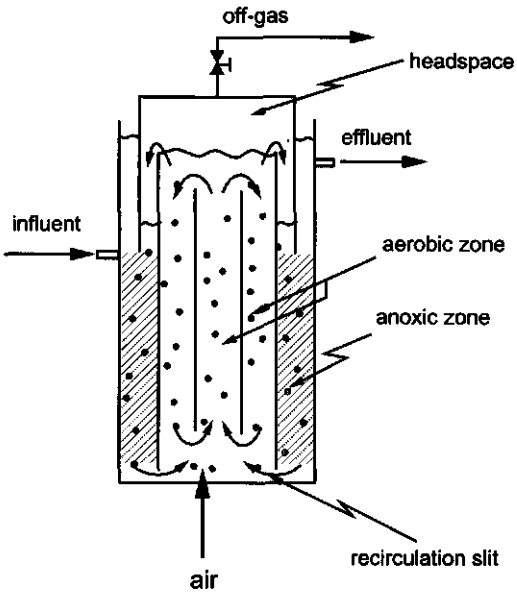


Figure 19 - The BASE reactor as proposed by van Benthum et al.[172].

Recirculation occurs through a hole at the base of the reactor that can be set wider or narrower according to the need of higher or lower exchange rate between the compartments. This control of the exchange rate will also depend on whether or not most organic matter is consumed in the anoxic part so that it will not reflect in decreasing nitrification capacity. Another important point is the need of a tight control of the air-flow because the flow should be sufficient to provide both good mixing and oxygenation, but at the same time should not be too high to prevent excessive aeration of the anoxic zone, which could decrease the denitrifying (and thus global) capacity of the system. This process is still under development, but so far it seems rather promising in effective removal of nitrogen compounds of high to medium loaded wastewaters.

Recently (1996), Frijters et al. [28] have reported on a study at pilot scale of a reactor concept (denitrifying CIRCOX®) very similar to that of the BASE just described. Much like the BASE, the denitrifying CIRCOX® is an air-lift reactor (nitrification, 2.28 $m^3$) that has been extended with an anoxic compartment for denitrification (1.16 $m^3$). Circulation between the two sections is made through a secondary air-lift pump (about 5% of the total gas-flow into the reactor, see Figure 20). The influent wastewater was fed into the anoxic compartment. The anoxic section behaved as plug-flow (average retention time 8 minutes) whereas the reactor as a whole could be considered well mixed (hydraulic retention time of 80-100 minutes).

Nevertheless, the authors showed that the concentration of the biofilm-covered carriers was constant throughout the whole reactor. Their pilot study was carried out during about one year with pre-settled municipal wastewater (67% domestic and 33 % industrial wastewater) at total-COD loads of 5-9 kg. $m^{-3}.d^{-1}$ and N-loads of 0.7-1.2 kg. $m^{-3}.d^{-1}$, and for temperatures that varied throughout the year between 8 and 24 °C.
The retention times were adjusted as necessary to maintain approximately the same the loads into the reactor as with rainy weather the average influent concentrations could fall down to one third of those under dry-weather conditions. Together with the denitrifying-CIRCOX reactor, a common CIRCOX (i.e. without anoxic compartment) was run as reference.

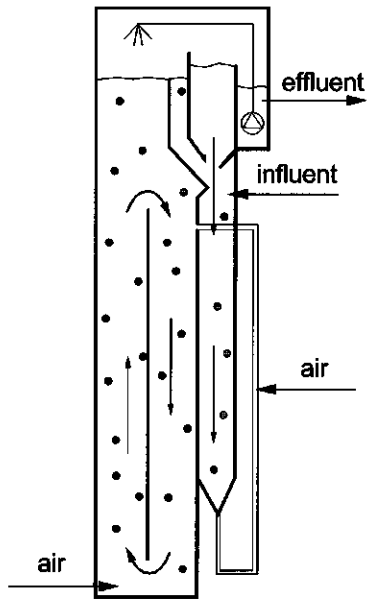


Figure 20 - Schematic diagram of the CIRCOX reactor according to Frijters et al. [28].

About 50 days after start-up, about 60% of the total ammonium load was converted (together with about half of the COD-load), whereas after 90 days most of the incoming ammonium had been removed together with 65 to 75 % of the COD loads. In their study, the authors reported also a strong reduction in nitrification rates at lower temperatures. This reduction, however, was considerably less important than that at the same temperatures (i.e., below 15 °C) for activated-sludge systems with equivalent dimensions (N-removal rates 20% higher). This reduced sensitivity to lower temperatures has been often observed in nitrification by immobilized (either artificially or naturally) cells, as it will be discussed in the next section. As in the BASE reactor,

control of the recirculation rate (provided by the air input in the recirculation pipe) is a fundamental parameter as it strongly influences the denitrification (and thus global) rates in the reactor. Specially for low COD-loaded influents, excessive oxygen may be present in the anoxic compartment and hinder denitrification if the aeration rates are not closely tuned with the oxygen consumption rates in the oxic zones. In general, the system was reported to function well except for C-loads than were lower than a certain minimum (> 2.5 kg COD. $m^{-3}.d^{-1}$), under which the biofilms became fluffy and tended to desegregate. Possibly, a minimum critical N-load may exist as well to guarantee the structure of nitrifying biofilms. Nevertheless, these results suggest that this system may be quite promising for the treatment of medium to high loaded municipal wastewaters.

BIOLIFT®

The company OTV (France) proposed another compact system (Biolift®) that basically consists of an activated-sludge tank that is placed on the top of two air-lift-type reactors (see Figure 21, [151,173]).

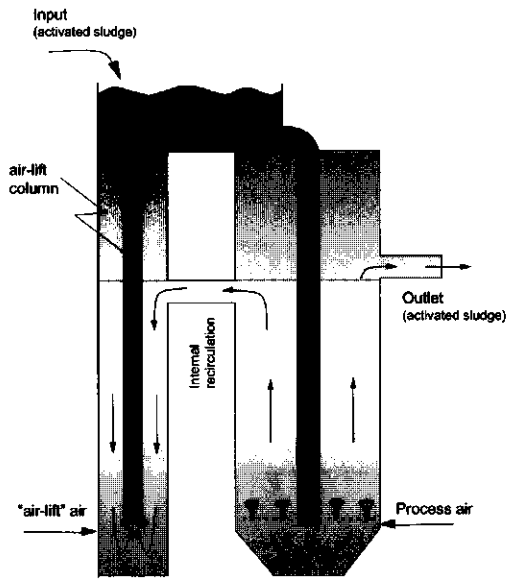


Figure 21 - The BIOLIFT process according to OTV [151].

Air is injected on the base of the air-lift column containing sand particles covered with biofilms. Nitrification takes place there. At the top of the reactor, the nitrified stream is mixed with the influent wastewater where organic carbon and part of the nitrification products are removed under anoxic conditions. This process continues during the

recirculation of the medium through another (larger) air-lift-like reactor, on the base of which process air is injected. Part of the medium passes then to the clarifier where sludge is separated from cleaned water whereas another part is recirculated to the nitrifying air-lift. According to the company, complete ammonia and nitrate removal rates between 3.5 and 4.5 kg per $m^3$ of settled media per day can be achieved with this process. The reported residence times are 3 to 4 hours for denitrification and less than one hour for nitrification. The Biolift® system has been in operation at industrial scale since 1993 [173]. According to the company, a nitrogen and carbon removal unit with a capacity for 10 000 p.e. requires an area of 40 $m^2$, has an effective volume of 230 $m^3$ and is 10 m high.

***Magic-beads and related concepts***

A rather different approach was proposed by Tramper [132,133] whose co-workers have been investigating the possibility of using gel beads with an outer nitrifying layer surrounding a denitrifying core for integration of nitrification and denitrification within one single biocatalyst.

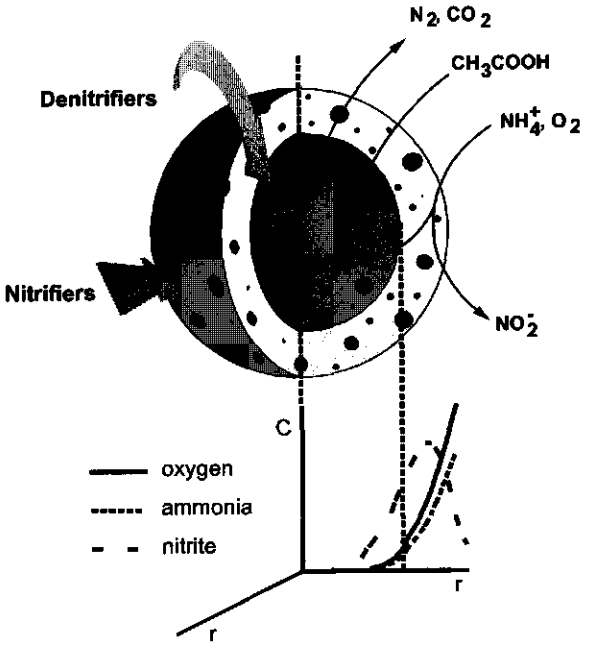


Figure 22 - The Magic-Bead Concept. Outer layer: nitrification; core: denitrification.

The biocatalyst particles are placed in a common air-lift reactor through which the waste streams can flow at almost any rate, without the need of recirculation to or from any anoxic compartment or reactor. The reasoning behind this rather compact, single system (proposed as the "magic-bead concept", see Figure 22) relies on the establishment of aerobic and anoxic zones within the cell supports as a result of oxygen diffusion limitation due to oxygen uptake by the nitrifiers [164,165,175].

The product of the first microorganism is the substrate for the second one, and thus this two-stage bioconversion process is conducted as if single staged. The biocatalyst particle is used optimally because both the external layers and core are active. In most immobilized-cell processes (based either in naturally or artificially immobilized biomass), just a relatively small part of the particle is used (eg., the outermost layers in aerobic processes) leaving the rest fully unexploited, as illustrated by the example in Figure 23. In the photograph, that shows a section of a fully nitrifying gel bead, the active biomass concentrated in a thin layer just underneath the surface despite the fact that it was uniformly distributed throughout the whole bead at the beginning of the experiment.

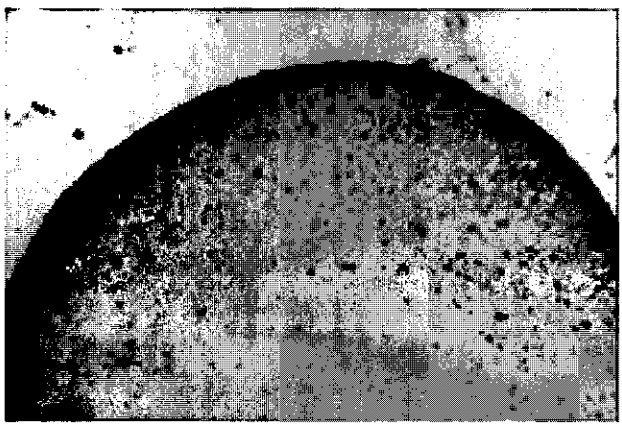

Figure 23 - Section of a carrageenan bead showing microcolonies of *Nitrosomonas europaea* after prolonged growth.

This process has been tested at bench scale with *Nitrosomonas europaea* and *Pseudomonas spp.* co-immobilized in κ-carrageenan. Martins dos Santos et al. [174,175] have demonstrated that coupled autotrophic nitrification and heterotrophic denitrification could be indeed achieved in 3 l air-lift loop reactors filled with 15-25%

(v/v) of such beads at rates up to 3-5 kg-N. $m^{-3}_{reactor}.d^{-1}$ for $NH_4^+$ concentrations ranging from 10 to 30 mM at 30°C. This reactor system performed positively both under steady state and dynamic conditions. Its behaviour under both conditions was well described by a dynamic model that predicted biomass and solute bulk concentrations, substrate consumption rates, product formation rates and both biomass and solute concentration profiles across the beads as a function of time [175]. Studies with specific microelectrodes for $O_2$, $NH_4^+$ and $NO_2^-$ showed that oxygen was indeed depleted after the first 100-300 μm, and that denitrification took place mainly beyond that distance (toward the centre) with no nitrate production (see Figure 24).

An important advantage of this system is that the nitrifying populations can be selectively immobilized and hence nitrite can enter directly into the denitrification route avoiding the intermediate steps with nitrate with considerable savings in energy and resources. Yet a third, and not less important benefit, is that the pH control is greatly facilitated because part of the acidity produced by nitrification is rapidly compensated see by the alkalinity resulting from denitrification. These effects (that make the system intrinsically much more stable and robust) have been quantitatively predicted by Sheintunch and co-workers [177] and Martins dos Santos et al. [178] (see also references [179,180,181]). These effects were also experimentally verified by Martins dos Santos et al. [178] using specific pH-microelectrodes together with the above mentioned nitrite, ammonium and oxygen microelectrodes.

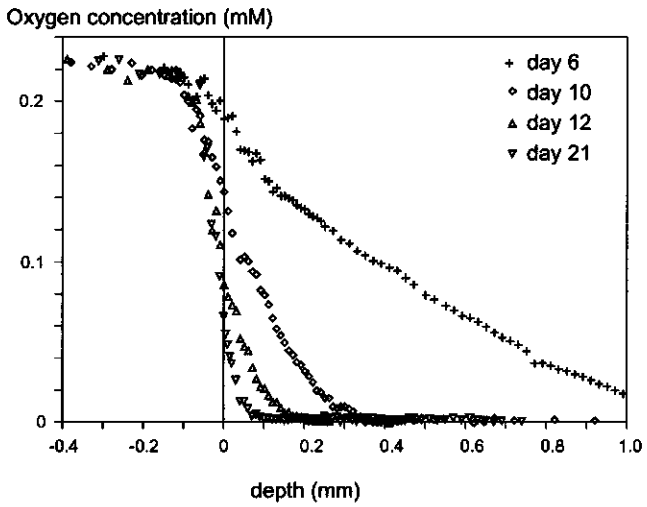


Figure 24 - Evolution of the oxygen concentration profiles along time in a support with immobilized nitrifiers. Adapted from reference [176].

Despite these positive results, in practice this system faces the problem of competition for oxygen and organic matter by heterotrophs present in the wastewater that may attach onto the bead surface. This problem has been addressed above for the MAL and its relevance will be discussed later in this section.

A similar process with nitrifying and denitrifying cells co-immobilized (as mixed culture) gel beads has been also presented by Kokufuta et al. [182,183] and Tartakovski et al. [177]. Their systems were based on the (expected) natural segregation of the nitrifying and denitrifying populations across the gel particles following the establishments of oxygen and substrate gradients throughout the beads. These systems were reported to function well in both batch and sequencing-batch, short-term experiments under aerobic conditions. Despite of these positive results, and although co-immobilizing both populations as a mixed culture is conceptually and technically much simpler, in practice this has demonstrated to be a difficult task, especially in the medium-long term. This is because the denitrifying cells are facultative denitrifiers that use nitrite or nitrate as electron acceptor only if oxygen is not available. Moreover, they grow much faster than nitrifiers and have higher affinity for oxygen. This all means that if both populations are simply mixed and directly immobilized in a gel bead, the denitrifiers easily outcompete the nitrifiers in the presence of a carbon source, even if their initial inoculum size is much smaller than that of the nitrifiers, as already addressed by Martins dos Santos et al. [164,164,174]. Indeed, Kurosawa and Tanaka had previously shown [184,185] that natural "habitat segregation" is only possible if the populations involved have considerably different substrate (oxygen) demands. Forced physical separation of nitrifiers and denitrifiers is thus a way of providing a (necessary) competitive advantage to the nitrifying cells [174,186].

The "magic-bead" concept as presented is in fact an idealized representation of what happens in naturally attached biofilms, in which the different microbial populations are distributed across the biomass layers according to the redox potential, oxygen availability and other environmental conditions prevailing at every point [39, 187-190] For instance, denitrification has been repeatedly shown to occur in the anaerobic zones of biofilms in aerobic trickling filters [191-193], rotating biological contactors [59,194-195] and other fixed-film reactors [196], in large flocs of activated sludge, in oxidation ditches [197] or simply in submerged soils [198] or sediments [199-201].
Recently, Hao and Nieuwstad [150] have studied the feasibility of coupling nitrification and denitrification in biofilms attached to carriers in a single air-lift loop reactor for the treatment of municipal wastewaters. The reactor removed 70-80 % of nitrogen loads

(TKN) ranging from 0.4 to 0.8 kg-N. $m^{-3}_{reactor}.d^{-1}$, and concomitantly eliminated 70-80% of 1 to 2 kg-COD. $m^{-3}_{reactor}.d^{-1}$ in the influent. Both the nitrification and denitrification rates were roughly proportional to the total biomass concentration in the reactor up to 10 $kg.m^{-3}_{reactor}$, after which they remained constant (up to a maximum of 30 $kg.m^{-3}_{reactor}$). The 200 l reactor, loaded with 50 kg. $m^{-3}$ small basalt carriers (0.2-0.3 mm, specific gravity 2.7 $kg.m^{-3}$) was operated for 120 days at temperatures that changed seasonally between 13 and 21°C. As the oxygen concentration throughout the whole reactor never came below 2 g $O_2$. $m^{-3}$, they explained these results by considering that the different biomass populations stratified according to the oxygen gradients across the biofilm-covered particles (see Figure 25). Organic matter was oxidized in the outermost layers of the biofilm whereas in the layers beneath nitrifying microorganisms converted ammonium into nitrite and nitrate at the expense of the oxygen left. Denitrification took place in the anoxic layers underneath. They referred the tight control of dissolved oxygen concentration as one of the most important parameters to guarantee a successful integration of nitrification and denitrification.

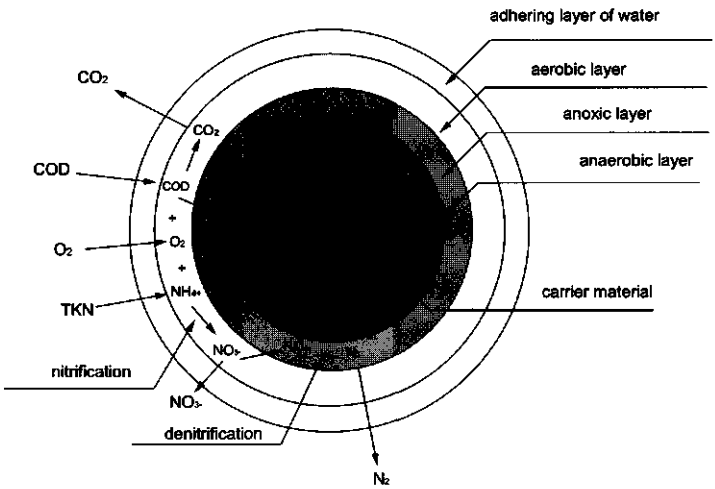


Figure 25 - Biofilm particle according to Hao & Nieuwstad [150].

This system is basically the biofilm version of the "magic-bead" concept above presented. An evident problem in both versions is that heterotrophs (either denitrifiers or other that will undoubtedly attach onto to the carrier surface) will always use as much oxygen as possible to consume the largest amounts possible of organic matter. If too much oxygen is consumed nitrification will proceed slowly (and hence denitrification)

due to lack of oxygen. Additionally, if too much organic matter is oxidized aerobically, there may be too little left to allow effective denitrification. These problems have been addressed above (see MAL) and demand a careful and tight control of, not just oxygen, but also of the flow rates applied and influent concentration of organic matter because these will ultimately determine the thickness and density of the heterotrophic layer covering the nitrifying region (see also references [174,186]). As these factors play against each other, the optimum nitrogen removal rates attained will be always a result of a compromise and doubtless below the maximum possibilities of the system. Ideally, such a magic-bead system (either in biofilm or artificially immobilized form) would proceed entirely autotrophically, without the interference heterotrophic growth. Such a process has been developed very recently and is based on the co-immobilization mixed cultures of common nitrifiers and strict anaerobic ammonium oxidizers (Anammox), that are entirely autotrophic [202-204]). The process is described below in the section "Novel processes".

***Membrane reactors***

*Membrane-separation bioreactors*

Since the last decade, membrane bioreactors have been steadily gaining importance as effective alternatives in the treatment and detoxification of industrial wastewaters [205,206]. Membrane units coupled to biological wastewater treatment units were used for the first time by Smith et al. in 1969 to separate and recycle biomass from the final effluent in activated sludge processes [207]. Since then, membrane-based treatment processes evolved considerably and the number of applications of such technology increased enormously [205,208]. Though, most of these applications have been still developed for separation and recycling of biomass from and into a common activated-sludge bioreactor (see Figure 26 [205,208]).

Membranes prevent loss of biomass and high-molecular-weight solutes from the reactor system, enabling thus the setting of hydraulic retention times that are independent of that of biomass. In this sense, a membrane-coupled bioreactor works much like an immobilized-cell system. Typical biomass concentrations in these systems range between 20 and 120 $kg.m^{-3}$ in aerobic and anaerobic processes, respectively, with hydraulic retention times ranging from 1.8 to 200 hours. Tubular, hollow-fiber, and plate-and-frame membrane configurations with pore sizes ranging from 0.1 to 0.3 μm, have given membrane fluxes (that thus determine the retention times in the whole system) from 2 to 70 $l.m^{-3}.h^{-1}$ (see reference [205] for a more extensive description).

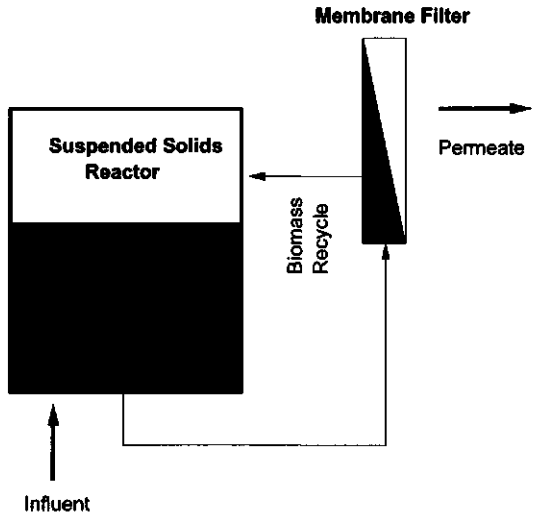


Figure 26 - Solid-liquid membrane separation reactor.

In these membrane-bioreactor configurations, the target compounds are metabolized primarily in the treatment tank (where most of the biomass is present) and not on the membrane itself, which is only a small part of the total bioreactor. This system easily enables thus either aerobic or anaerobic treatment but it makes it difficult to achieve integrated aerobic-anaerobic bioconversions. This means that such systems have been commonly used either for nitrification [209,210] or denitrification [211,212] but less frequently for integrated nitrogen removal as such. To provide conditions for nitrification and denitrification, strategies similar to those with single tanks or compartments have been used. For instance, Chiemchaisri et al. [213] applied intermittent aeration (90 minutes cycles) to a suspended-solids bioreactor with incorporated hollow-fiber membranes so as to obtain 87% organic removal, 87% nitrification and 84% denitrification from domestic wastewater in steady-state for about 330 days. The removal of nitrogenous compounds was shown to be a function of the BOD/total Kjeldal ratio in the influent [214]. Similar configurations have been applied at both pilot and full scale for the treatment of waste streams from landfill leachates with nitrogen removal rates up to 0.7 kg-N.$m^{-3}$.$d^{-1}$ [94,208,215]. Due to the relatively small footprint of membrane-separation bioreactors (and to the relatively high costs involved, see below), most of the current applications of these systems at full scale are in operation in industrialized countries where space is more acutely a problem, such in the [Netherlands 208] and Japan [216,205] or in densely populated areas in the U.S.A [95,217] or Germany [215].

*Membrane-based biodegradation*

In an attempt to combine the advantages of membrane-based technology (retention and separation of biomass from clean effluent after treatment) with those of immobilized-cell technology (high biomass concentrations, large specific areas for mass transfer), several groups developed other membrane-reactor concepts in which biodegradation occurs on the membrane itself rather than in a separate reactor section. Based on the observation that both nitrification and denitrification are often hampered by aerobic heterotrophic carbon oxidation (though by different reasons, see above), Timberlake et al. [218] proposed what they called a Permeable-Support Biofilm (PSB), in which air or oxygen is supplied through a permeable membrane support (TEFLON membrane sandwiched between two layers of nylon wooden fabric) that is placed in an agitated tank sparged with nitrogen (Figure 27).

Due to the existence of simultaneous aerobic and anoxic zones and by controlling the amount of organic matter supplied, a structured biofilm developed naturally in such a way that nitrifiers attached directly onto the membrane whereas the denitrifiers and other heterotrophs formed layers attached to those of nitrifiers and in close contact with the anoxic, carbon-reach medium. Heterotrophic carbon oxidation, nitrification and denitrification take thus place in different parts of the membrane-biofilm (see scheme in Figure 27).

Although both the nitrogen removal rates attained (about 0.05 kg-N.$m^{-3}$.$d^{-1}$, for influent ammonia concentrations of 24 mg-N/L) and the specific surface (20 $m^2_{membrane}/m^3_{reactor}$) were rather low, this membrane-reactor concept is innovative and present some potential advantages especially if the specific surface area is increased (see further discussion on this in the next section). In this system optimal conditions for nitrification, denitrification and organic-carbon removal are provided to the different populations involved without interfering with one another, avoiding thus competition and possible inhibition problems such as those described above (see MAL subsection).

Later, other groups, such as those of Hirasa et al.[219] and Suzuki et al [220] reported on similar constructions with comparable nitrogen removal rates using silicone and Teflon membranes, respectively. Lemoine et al.[221] have used series of membrane modules made up of an agar layer entrapped between two microporous membrane filters to denitrify spring water at rates ranging from 1.2 to 2 kg-N.$m^{-3}$.$d^{-1}$. They claimed that no biomass leaked into the effluent drinking water. This membrane-reactor concept was further explored by Özoguz et al. [222] who presented a bench-scale 22 l membrane reactor that consisted in a silicone membrane densely coiled and packed within a cylindrical frame filled with the bulk medium (maintained anaerobic). Oxygen was

supplied under a pressure of 3 atm through the permeable membrane that had an exchange specific surface of 243 $m^2/m^3$.The reduced substrates ammonium (34 mM) and acetate (17 mM) were supplied at a retention time of 4.2 hours to the bulk liquid that was recycled continuously at a rate of 2000 liters/h. Temperature was 26.5 °C. The biofilm grew on the tubing with a thickness between 2 and 3 mm. Under these conditions, up to 80 % of the total influent N was fully mineralized at a rate of 2-2.2 kg-$N.m^{-3}.d^{-1}$, which is comparable to the rates attained in air-lift and fluidized-bed systems described above (see also Table 2).

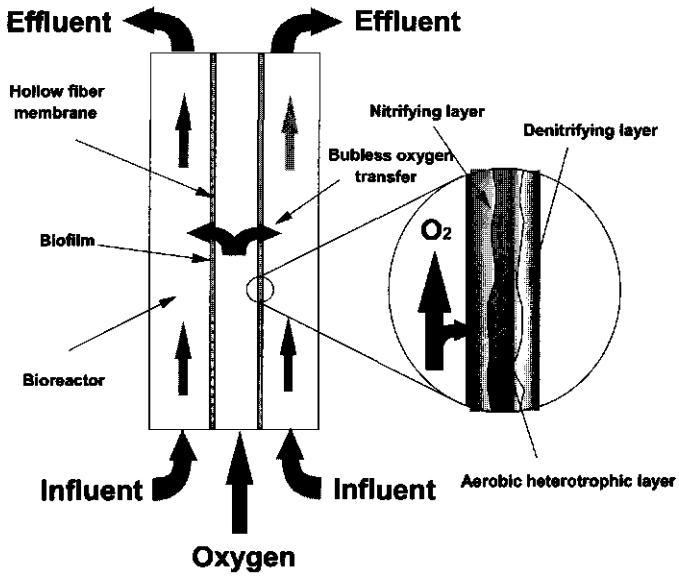


Figure 27 - Permeable-support biofilm [205,218].

By using specific microelectrodes (tip diameter 10 to 15 µm) for ammonium, oxygen, nitrite and nitrate, de Beer and colleagues[223] demonstrated that nitrification took place chiefly within the first 150 µm from the outer surface of the membrane (which also corresponded to maximum the oxygen penetration depth) whereas denitrification occurred mainly beyond that point. Nitrite (not supplied in the influent feed) was produced in the oxic zone and its accumulation was the highest where nitrate consumption occurred, i.e., just below the oxic section. Such a nitrite peak was observed in this nitrifying zone but not in the anaerobic denitrifying zone. These observations agreed well those of Schramm et al. [224] that used in-situ hybridization techniques to reveal the structured distribution of the different populations (nitritifiers,

nitratifiers, denitrifiers and other heterotrophs) across the biofilm. Such distribution matches that shown schematically in Figure 27, proposed previously by Timberlake et al. [218].

A similar, though slightly different concept is that presented by Uemoto and Saiki [225,226], in which the membrane-support was a 125 mm hollow-tube ($Ø_{int}$=5 mm, $Ø_{ext}$=12 mm) made up of a polymeric gel where pure *Nitrosomonas europaea* and *Paracoccus denitrificans* were artificially co-immobilized. The tubular gel was placed in an aerated, stirred reactor filled with ammonium-containing wastewater. An organic, external C-source (ethanol) was pumped through the inside part of the tube that was maintained anaerobic. *Nitrosomonas europaea* concentrated in the outer layers of the gel oxidized ammonium into nitrite whereas *Paracoccus denitrificans* reduced nitrite to nitrogen at the expense of organic matter. The ethanol was recirculated through the tubular gel without coming in contact with the ammonium-containing wastewater, preventing thereby competition both for oxygen and organic matter between heterotrophs and nitrifiers. The maximum nitrogen removal rate attained in the 100 mL working-volume reactor was about 0.24 kg-N.$m^{-3}$.$d^{-1}$, for a retention time of about 6.7 hours at 30 °C. Using fluorescent-antibody labeling Uemoto and Saiki found that, although initially both populations were homogeneously distributed throughout the whole thickness of the gel (1 mm), with the course of time the nitrifiers tended to accumulate in a 50-150 μm layer underneath the outer surface of the gel, whereas the denitrifiers distributed further down to 300-400 μm. They proposed a three-zone distribution structure where in the first (0 to 100 μm) only nitrification took place, whereas on the second (100-200 μm) nitrification and denitrification occurred simultaneously and a third (beyond 200 μm) where only denitrification took place.

*Discussion on the use membrane reactors*

The simplest construction of those above described is that in which the membrane (either in the hollow-fiber or plate-and-frame version) is merely used for separation of biomass. Simplicity is an intrinsic, important advantage in any process and that is probably the reason why this is the only large-scale membrane-based configuration so far reported in nitrogen removal (see above). Nevertheless, its compactness is still limited and, since either only aerobiosis or anaerobiosis can be maintained in the tank at the time, coupled nitrification and denitrification is difficult to maintain in steady operation. The configurations in which the membrane itself supports growth and to which both aerobiosis and anaerobiosis can be applied can be thus an effective alternative, such as those of Özoguz et al. [222]. In these (not yet optimized) systems the nitrogen removal rates attained were considerably high and at the same time with no

important problems of competition for substrates by the intervening populations. This is especially important in a system such as that of Özoguz et al. (proposed by Timberlake et al. [218]) in which the ammonium-containing wastewater contains the carbon source as well (see discussion in MAL subsection). It is thus conceivable that such a system could be optimized to a membrane module consisting of a bundle of hollow-tubes fixed in a shell-and-tube arrangement, enlarging thereby the specific surface area. It is, however, unlikely that a truly, densely packed hollow-fiber module (with a fiber diameter of 50-300 μm) could be used because of the predictable clogging problems (see below).

Although membrane-based bioreactors enable high biomass concentrations and thus considerably higher removal rates than those attained in conventional activated-sludge systems, in general they tend to require much energy both to sustain biological activity and to maintain membrane performance. Chiemchaisri et al.[213] calculated that existing aerobic membrane-separation bioreactors applied to wastewater reuse systems used in large office buildings in Japan consumed power up to 3-5.5 $kWh.m^{-3}$ of permeate, which is about 10 times greater than that required by conventional activated-sludge processes. Indeed, the high-energy requirement of recirculation needed to maintain a high cross-flow velocity and simultaneously a good membrane permeability is a major problem hindering the widespread application of membrane separation processes in bioreactors. [There are, nevertheless, few reports on improvements on this point. For instance Yamamoto et al. [227] claimed that incorporation of hollow-fiber membranes directly into the suspended solids reactor (instead of placing it out of it for recirculation of biomass) reduced the operating costs down to levels comparable to those of activated-sludge processes].

An additional and important problem with membrane-based reactors is that the accumulation of biomass and solutes or other components on the membrane walls leads almost inevitably to clogging of the membrane and decrease of its permeability (and thus also flux of substrates and oxygen), which in turn demands more energy to maintain membrane performance. Furthermore, plugging of the membrane walls tends to reduce significantly the specific surface available for mass transfer and biomass activity.

Effective and periodic backwashing or even membrane washing is thus as necessary as in the above described biofilters. These two main drawbacks (together with the relatively high costs of the membranes themselves) have resulted in that most of the applications with membrane bioreactors involve recuperation of valuable components from the effluent streams (such as heavy metals [228]) or treatment of complex and

"difficult" wastewaters (such as oily wastewaters [95,206,217]), and are less frequently used in "more common" processes such as nitrogen removal. Though some commercial size membrane-based bioreactors exist for wastewater purification (almost exclusively membrane-separation bioreactors), and although intensive research is carried out on the improvement of membrane operation, reduction of the energy requirements and prevention of fouling and clogging, much remains to be done before membrane reactors can be actually used in common wastewater nitrogen removal processes.

**New concepts on combined nitrification and denitrification**

All the nitrogen removal processes described above implicitly assume that chemolithotrophic, strictly aerobic bacteria accomplish the bulk of nitrification whereas facultative heterotrophic microorganisms carry out the denitrification steps under anoxic conditions. Because of that, virtually all the systems devised rely on either the establishment of both aerobic and anoxic conditions within the same reactor or support, or on the use of separate reactor (compartments) to attain complete nitrification and denitrification. However, during the last decades several new principles on nitrogen removal have emerged and some other were rediscovered, which makes this strict division on autotrophic nitrification and heterotrophic denitrification too rigid and limited. These principles include heterotrophic nitrification, aerobic denitrification, nitrifier denitrification, anaerobic oxidation of ammonium, (de)nitrification chiefly via nitrite, and also combinations of these and the "classical" nitrification/denitrification processes. A short survey of these principles and their relevance for wastewater treatment is given below. For a more detailed and complete description of these aspects, the reader is referred to the reviews of Jetten et al. [229,230], Kuenen and Robertson [231], Robertson and Kuenen [232] and Cole [10,233].

***Heterotrophic nitrification***

Although most of nitrification processes are carried out by chemolithotrophic microorganisms, there is a large variety of heterotrophs also capable of nitrification, provided that a suitable organic energy source is present [230,234,235] These microorganisms, however, seem no to extract energy from nitrification. Heterotrophic oxidation of ammonia is thought to occur as a means of dumping excess of reducing power [1,230,231,235,236]. Given the dominance of heterotrophs over autotrophs in most (eco)systems, these observations imply that heterotrophic nitrifiers (of which a large number have shown to denitrify aerobically, see next section) could in principle contribute significantly to nitrification in nature and water treatment plants. In fact, the combination of the heterotrophic nitrification properties of such heterotrophs with those

of aerobic denitrification would suggest that these microorganisms could be used for single-stage nitrogen removal in wastewater purification systems, particularly under conditions non-favorable to the "classical" nitrifiers. Indeed, Van Niel and colleagues [237] reported nitrification at rates comparable to those of autotrophic nitrification in competitions experiments with substrate-limited chemostat mixed cultures of *Ns. europaea* (autotrophic nitrifier) and *Thiosphaera pantotropha* (an heterotrophic nitrifier). Also Gupta and co-workers [238] have successfully used a mixed culture of *Ns. europaea* and *T. pantotropha* to treat wastewaters using a RBC process (see subsection RBCs). Daalsgaard et al. [56] studied in detail the growth of *T. pantotropha* in such biofilms and showed, by using a combined microelectrode for $O_2$ and $N_2O$, that indeed heterotrophic nitrification was quantitatively very important in the whole nitrogen conversion process. Seyfried and co-workers [239] report to have applied this same concept of heterotrophic nitrification coupled to aerobic denitrification and reported a total removal efficiency (i.e., from $NH_4^+$-N to N-gases) of 90% for a nitrogen load of 2 g-N.$m^2$.$d^{-1}$ (average inflow of 65 $m^3$.$d^{-1}$).

Despite the reported successes and the promising ideas, a general problem with a process using heterotrophic denitrifiers such as that above described is that ammonium and nitrite tend to be assimilated instead of mineralized, especially for higher C:N ratios [234,237]. Because of this too much biomass is produced, which makes the process unsuitable for efficient nitrogen removal in most wastewater applications. Besides, in practice, most wastewaters contain (either intrinsically or after passing a COD-removal stage) relatively low COD:N ratios, conditions under which autotrophic nitrification is clearly favored. Such microorganisms are thus only likely to be suitable for bioremediation purposes in conditions under which common nitrifiers are poorly active e.g., acid soils or wastewaters, or at higher temperatures [240]. Another potential drawback (that will be further considered in the next subsection) is that heterotrophic nitrifiers (much of which are aerobic denitrifiers as well) tend to form, under certain conditions, nitrogen oxides together with molecular nitrogen as result of the reduction of intermediate nitrogen compounds (nitrite, nitrate or hydroxylamine) [230,241,242]. Altogether, these drawbacks result in that the use an heterotroph denitrifiers such as *T. pantotropha* may not be adequate for treatment of waste waters. A feasibility study of such a system has confirmed this unsuitability [243].

***Aerobic denitrification***

As just mentioned, aerobic denitrification is often intimately associated to heterotrophic denitrification [232,241]. The aerobic denitrification rates, however, are much lower

than those under anaerobiosis. All the aerobic denitrifiers thus far studied increased their denitrification rate as the dissolved oxygen concentrations in the culture fell [1]. Hence, under the conditions that commonly prevail in treatment plants, the bulk of denitrification is in general simply attributed to "classical" denitrification. Likewise heterotrophic nitrification, the issue about aerobic denitrification (as well as its coupling with heterotrophic nitrification) is not so much its (limited) potential for nitrogen removal but more the problems it brings. Indeed, aerobic denitrification is often an incomplete process by which intermediaries as NO or $N_2O$ accumulate instead of $N_2$ [235,244-248]. The conditions under which these undesirable intermediates are produced are not yet entirely clear but it appears that the NO and $N_2O$ reductases may be particularly sensitive to environmental stresses such as sub-optimal pH, temperature, unbalanced C/N ratios or fluctuations in the oxygen concentrations [235,241,249-257]. For more detailed information, the reader is referred to the reviews of Ferguson [258], Kuenen and Robertson, [231], Jetten et al [230], Payne [259], Knowles [260], and the works of Anderson & Levine [252], Davies et al [44], Hochstein et al [245], Alefounder et al [261].

Clearly, these studies have serious implications to wastewater treatment. In fact, recent reports show that a significant part of the biogenic $NO_x$ release into atmosphere comes from sub-optimally functioning wastewater purification plants [254,262,263]. This is, in a sense, not too surprising, if taken into account the results just reported. In fact, most of the conventional nitrogen removal systems described above involve either recirculation of biomass between aerobic and anoxic zones, or the application of alternate aerobic and anoxic conditions. Since several of the "new" treatment systems also involve these kind of configurations, much care should be taken to avoid amplification of the $NO_x$ emissions. Indeed, $N_2O$ emissions (for not clearly identified reasons) have been already reported in some of these newly developed systems [152,172,174] This illustrates furthermore an additional problem, which is that in the vast majority of plants, possible formation of nitrogen oxides is not even followed. This means that the importance and occurrence of these unwanted emissions is often strongly underestimated. Hence, besides the inventive, intrinsic development of novel technologies for nitrogen removal, to their effective and safe application in actual treatment plants, it is first essential to: a) gather as much as possible information of all possible side-effects of the system, including monitorization of all possible sub-products; b) obtain more structural and kinetic insight into mechanisms of action of the enzymes involved and the way environmental factors affect their functioning and thus of the system as a whole; and c)

control the operating conditions very tightly so as to avoid formation of these denitrification products.

***Nitrifier denitrification***

Although autotrophic nitrifying cells are generally considered to be strict aerobic autotrophic bacteria, under certain circumstances, they can be grown as heterotrophs while denitrifying. These properties are important for wastewater treatment because in most reactor configuration-s there are zones depleted of oxygen and under these conditions both *Nitrosomonas* and *Nitrobacter* strains are able to denitrify as well as nitrify, although at much lower rates than aerobic nitrification or "classical" heterotrophic denitrification, respectively [3,264-266]. Again, more than a benefit, these "alternative routes" pose generally the problem that the products of this "nitrifier denitrification" are also often $N_2O$ and NO[230,231,268,269]. In many cases, these reactions seem to be mechanisms by which the autotrophic microorganisms can cope with oxygen limitation or even with anaerobiosis [238]. Indeed, several studies indicate that the $NO_x$ emissions are closely related to the oxygen concentrations they are submitted to [231,248,251,264-270]. Under low oxygen tensions, autotrophic nitrifiers appear, in some cases, to be able to use alternative routes in which ammonium, hydroxylamine or even hydrogen may serve as suitable electron donors for nitrite reduction [265,269,271-274].

Thus, and despite the fact that sometimes part of the ammonium can be directly converted in dinitrogen the relevance of nitrifier denitrification for real wastewater treatment and the arguments for its tight control are much the same as those referred in the last subsection, in which it was shown to be more frequently undesirable than wanted. Thus again, a thorough understanding of the underlying mechanisms is essential to prevent unwanted emissions and especially for not amplifying these (due to increased throughput capacities) with respect to conventional treatment systems.

***Nitrite-route***

As discussed earlier in the sections *Demands on nitrogen removal* and *Magic-beads and related concepts*, a straightforward (potential) advantage of combining nitrification with denitrification in a single, compact system, is that two "superfluous" steps could be avoided. In nitrification nitrite is oxidized into nitrate at expenses of oxygen, whereas in the first denitrifying step nitrate is reconverted into nitrite while consuming organic carbon. By letting denitrification proceed directly from nitrite, about 25% oxygen and up to 40% organic carbon could be saved. These savings in oxygen and carbon would

thus be reflected on a reduction of the running costs connected both to aeration (energy) and raw materials (less external C-source needed in wastewater poor in organic carbon). Despite the clear advantages of denitrification via nitrite, in practice, however, and except for processes involving artificially-immobilized pure cultures of ammonium-oxidizing cells (see references in *Magic Beads and related concepts* above, and Wijffels et al. [135], 1994, Hunik et al. [23] and references therein), nitrification proceeds almost always to nitrate, even in biofilms where nitrifiers and denitrifiers coexist closely [59,189,190,194,275]. Several, not always successful attempts, were made in the past to direct nitrification towards nitrite build-up (in a step prior to denitrification) by inhibiting nitrite-oxidizing bacteria in several ways (see for instance references [276-279]).

Recently, alternative concepts have shown promising possibilities. For instance, it had been long known that although at low temperatures nitrite oxidizers grow faster than ammonia-oxidizers, above 15°C the ammonium oxidizers grow quicker [229,230]. This means that at higher temperatures and relatively high ammonia concentrations, it could be possible to wash out nitrite oxidizers in a system without sludge retention if the system is run under wash-out conditions [230]. Such concept had been suggest by Abeling & Seyfried [277] and was further developed by the group of Kuenen (SHARON process, Brower [280] as referred by Jetten et al. [230]). By applying dilution rates lower than 1-1.2 $d^{-1}$ (no sludge retention) to a chemostat operated at temperatures ranging from 25 to 40°C, they were able to get an effluent with virtually no nitrate and from which practically all nitrite oxidizers had been washed away. By applying discontinuous aeration to this reactor, it was possible to obtain stable nitrification and denitrification within the same reactor with little energy requirements. They report that, although the reactor was approximately three times larger than a common nitrifying reactor alone (with sludge), the total treatment volume did not increase because no settling tank and no sludge recycle were needed.

Garrido et al. [267]have recently proposed to manipulate the oxygen concentration in a biofilm-based reactor system as a way to achieve nitrite build-up. Their proposal relies on the observation that at low oxygen tensions the rate of nitrite reduction seems to decrease more than the rates of ammonia oxidation, which ultimately leads to nitrite accumulation (see also [110,111]). Indeed, in their experiments using a continuous biofilm reactor operated at low oxygen concentrations, Garrido et al. [267]) reported full ammonium conversion (5 kg-$N.m^{-3}.d^{-1}$) with a maximum of about 50% nitrite and 50% nitrate in the effluent. Short-term experiments with their biofilm particles have shown

that nitrite build-up was maximum (50% of all ammonium converted without significant loss of the ammonium oxidation capacity) for dissolved-oxygen concentrations of 1-2 $mg.l^{-1}$. Their results were reasonably described at reactor level by models based on biofilm kinetics and on the difference in the oxygen affinity constants for ammonium and nitrite oxidizers [267,281].

Although the reasons for the described behavior are not fully clear yet, Garrido and co-workers seemed to suggest that the explanation would rely on the difference in the affinity for oxygen of ammonia and nitrite oxidizers. Such differences have been indeed reported in literature (see eg. references [110, 221,277], but their exact their values vary widely. Also, the magnitude of these affinity differences is, in absolute terms, relatively small (see also Hunik et al. [23]). Thus, if the difference in the affinity for oxygen is indeed the reason underlying nitrite build-up in a mixed culture at low oxygen tensions, a system that relies on it alone would have a relatively narrow range of operating conditions an would require a rather tight control. More detailed studies on the mechanisms underlying nitrite-build as well as on long-term stability seem thus necessary prior use of the proposed system for effective nitrogen removal.

***Autotrophic Anaerobic Ammonia Oxidation (Anammox®)***

Until recently, and with the exceptions mentioned in the *Nitrifier denitrification* section, the bulk nitrification considered as such (either autotrophic or heterotrophic) was thought to occur strictly under aerobic conditions. Few years ago, however, a novel and radically different nitrogen removal process was discovered in which ammonium is converted directly into dinitrogen gas under anaerobic conditions with nitrite or nitrate as electron acceptor and in the absence of any organic C-source (European patent 03271841, [202,203,282,283]). See also the reviews of Robertson and Kuenen [1], Kuenen and Robertson [231] and Jetten et al.[230]. That such an autotrophic anaerobic ammonium oxidation process (Anammox®) should exist was already predicted theoretically (on thermodynamic grounds) by Broda in 1977 [284].

The process has now been demonstrated to exist and to be mediated biologically, although the identity of the responsible microorganism(s) has not been unraveled yet [202,203]. Yet, preliminary results seem to suggest that a single microorganism may be the responsible for the process [203]. Strous et. al [285] have recently shown that the Anammox sludge is reversibly inhibited by oxygen (i.e., although it stops its nitrogen removal activity under aerobiosis or microaerobiosis, it recovers activity as soon as oxygen is removed).

The Anammox process as found by Kuenen and co-workers (with nitrite as electron acceptor) is given by the following energy-synthesis equation developed on the basis of elemental balances and the experimentally determined yields of biomass on substrate [203]:

$$NH_4^+ + 1.31\ NO_2^- + 0.0425\ CO_2 \Rightarrow$$
$$1.05\ N_2 + 0.22\ NO_3^- + 1.87\ H_2O + 0.09\ OH^- + 0.0425\ CH_2O \qquad (1)$$

This process was tested in a fluidized-bed reactor fed with anaerobic, synthetic mineral medium containing ammonium and nitrite [203,230]. Within three months of operation (retention time 4.2 hours, 30°C, pH 7) the biomass in the reactor (attached onto fine sand particles) was able to convert up to 3 kg N. $m^{-3}_{reactor}.d^{-1}$ on a feed of 30 mM ammonium and nitrite. These results clearly show the enormous potential of this process for wastewater treatment [229,230].

Two possible problems in this process, however, are a), the very low growth rates of the responsible microorganisms ($\mu_{max} \approx 8*10^{-7}\ s^{-1}$[202,203]), the need of a continuous, roughly equimolar, supply of nitrite to the system for effective ammonium removal. Immobilization is likely to provide a way to circumvent the problem posed by the slow-growing microbial cells, as it was demonstrated by the use of attached biomass in the fluidized-bed above.

For the continuous supply of nitrite as electron acceptor in the oxidation of ammonium (avoiding nitrate, that seems to be converted first to nitrite before it is used into ammonia oxidation [203]), a straightforward strategy would be to combine this process with that of nitrification via nitrite. Roughly, the stoichiometric equations for the transformation of ammonium are (to improve clarity, no biomass synthesis is included):

Common nitrification:

$$NH_4^+ + 1.5\ O_2 \Rightarrow NO_2^- + H_2O + H^+ \qquad (2)$$

Anammox:

$$NH_4^+ + NO_2 \Rightarrow N_2 + H_2O + OH^- \qquad (3)$$

Nitrification plus Anammox [(2)+(3)]:

$$2\ NH_4^+ + NO_2 \Rightarrow N_2 + 3\ H_2O + OH^- \qquad (4)$$

Such an approach has been tested (and patented)by coupling the SHANNON process above described in series with that of the Anammox. In preliminary experiments, overall conversion ranged 0.4 to 0.8 $kg.N.m^{-3}.d^{-1}$ (80% of 40 mM influent ammonium, [285,286]). Similarly to a conventional nitrogen removal system, these reactors (SHANNON plus Anammox) can be placed in series in various combinations, either with or without recirculation between them, depending on the specific needs of the wastewater to be treated. Since the Shannon process (suspended-cells) operates better at moderately high dilution rates (2-0.5 $d^{-1}$[229,230]), the intrinsic advantages of an immobilized-cell system (retention of biomass, high throughputs) can be adequately exploited as well.

Another attractive possibility for supplying the nitrite needed by the "Anammox cells" would be applying the "Magic-bead concept" to the process. Such an application is schematically shown in Figure 28. Ammonium present in the waste streams flows into to the beads where in the outer layers is partly oxidized by the immobilized ammonium oxidizing cells. Due to the fast oxygen uptake by the nitrifiers, both biomass and substrate profiles develop leading to the establishment of extensive anoxic zones within the beads.

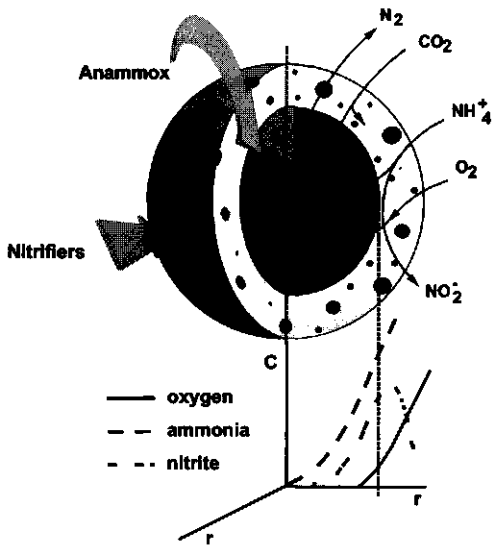


Figure 28 - The Magic-Bead Concept applied to the Anammox. Outer layer: aerobic nitrification; core: anaerobic ammonium oxidation.

The nitrite produced by the aerobic nitrifiers and the remaining ammonium (preferably half of the total) are then anaerobically converted into nitrogen by the Anammox microorganisms entrapped in the bead core. In this way, the whole process is single-staged and made almost as much compact as it can be. The system was shown to work both in batch and in the continuous mode. Overall nitrogen removal rates up to 1.5 kg-$N.m^{-3}.day^{-1}$ were achieved in continuously-driven air-lift loop reactors (15 mM $NH_4^+$ in feed, 20% v/v beads, 4-5 mm bead diameter [204], see Figure 29). Since the Anammox sludge is autotrophic and strictly anaerobic, no competition for oxygen with the nitrifiers would be expected. Therefore, the system was further simplified by mixing both nitrifying and Anammox sludge and by immobilizing them together in single beads. Such simplified configuration performed as well as that with segregated populations. The interactions between nitrifying and Anammox microorganisms, their growth, conversion of substrates and mass transport throughout the beads (both single and double-layered) was well described by a mathematical model, both under steady and dynamic conditions [287]. Besides the insight given by the model into mechanisms underlying the processes involved, it proved to be a very useful tool for the design of experiments and for optimization of the process. Yet, for the application of this co-immobilized Anammox-nitrifier system in practice, a suitable carrier material should yet be found [287,288].

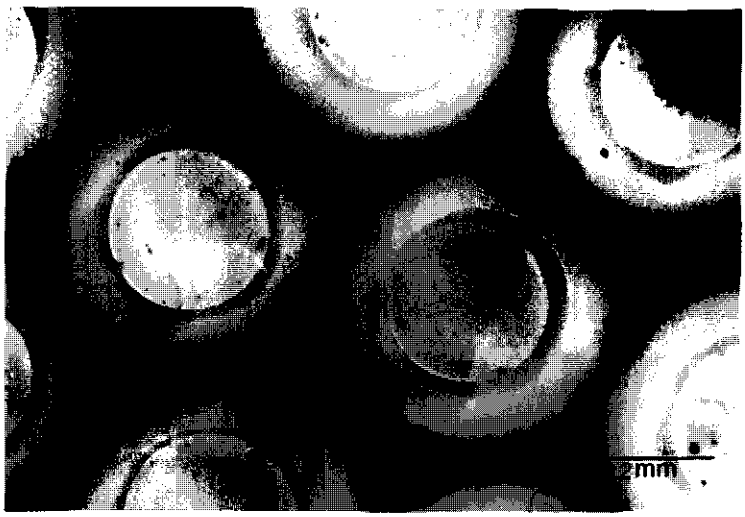


Figure 29 - Photograph of "Magic beads". Average diameter : 4.3 mm (Reproduced from reference [186] by permission of Elsevier Science Inc.).

These two systems (Shannon-Anammox and Magic-Bead - Anammox) present, besides their compactness (especially of that based on "magic-beads"), the advantage of being fully autotrophic. In addition to considerable resource savings, the absence of organic-carbon in the influent medium gives much more stability to such a reactor system because it prevents heterotrophic growth of microorganisms present in the wastestreams. Also, the supply of oxygen (and thus the energy costs) can be largely reduced because half of the ammonium is oxidized anaerobically. Since Anammox produces base, the acid formed during the aerobic oxidation of ammonia will be almost completely neutralized, facilitating thus greatly the pH control. Finally, being both populations very slow autotrophic growers, sludge production is negligible, reducing enormously the disposal of biomass surplus. In a sense, these systems can be considered almost perfect (in particular for highly loaded wastewaters) because they fulfill all the requirements of *Future demands* discussed in the corresponding section (see table 1). Yet, thorough technical-economical feasibility studies should be done prior application of these processes at full scale.

**Biofilms versus artificially immobilized cells**

From the analysis of all the systems above described for nitrogen removal, it is clear that the large majority of the so called *New Generation Bioreactors* are based on immobilized cell technology. Part of them are based on naturally attached biomass and part on artificially immobilized cells. In both types of systems the cells experience the same restrictions on substrate and product transport over the liquid-solid interface and throughout the immobilization matrix. The growth process, however, is different. In gel beads it begins by an homogeneously distributed (defined) biomass that ultimately concentrate underneath the bead surface, whereas in natural biofilms growth consists of the accumulation of cells on the surface of an initially bare carrier (see Figure 23).

Because of these differences, both types of systems will behave differently as well. For instance, whereas growth on an artificial matrix leads to increased cell density maintaining the dimensions of the bead, growth in natural biofilms leads to increased volume and thus to decreased specific surface area of the biocatalyst particle [35]. In steady-state, biofilm growth in natural biofilms is fully counterbalanced by detachment, which in turn depends primarily on the shear forces acting on it [39,290-293]. In artificially immobilized-cell processes cell detachment as such does not occur if the carrier is mechanically stable itself. Nevertheless, several authors [137,295,296] have shown that cells do erupt into the surrounding medium as a result of colony excretion. Thus, in both types of systems free cells will, sooner or later, appear in the effluent.

In addition to these general aspects, there are differences between both types of systems that strongly affect not only their behavior toward nitrogen removal, but also their technical and economical feasiblity for application at full scale. Presently, the main disadvantages of artificially immobilized with respect to biofilm systems are:

a) The immobilization procedure is more demanding, both economically and in labor, than that of biofilms. Indeed, natural immobilization is in principle relatively simple to obtain in biofilm-based systems providing that the hydrodynamic conditions are appropriate and the support is a little rough (see also Heijnen et al [16]).

b) The raw materials used for artificial immobilization (either natural or synthetic polymers) are generally more expensive than the carriers used in biofilm systems (sand, basalt, silicates, etc.). However, and although this is intrinsically so, a widespread use of artificially immobilized-cell systems would obviously decrease much the immobilization costs.

c) Thus far, most of the materials used for cell entrapment in nitrogen removal processes were natural gels such as κ-carrageenan or alginate. These materials are very mild for cell immobilization but are clearly unsuitable for practical application to this or any other wastewater treatment process because they lack mechanical stability, depend on a counterion to maintain its structure and/or are biodegraded by the microorganisms present in waste streams [294,288]. On the contrary, materials such a basalt, sand or activated carbon are both inert and stable, and are obviously not biodegradable. With respect to this, however, in the last few years several synthetic polymers were developed that are simultaneously very stable and not too toxic for the populations involved. Some of these polymers have been applied in nitrogen removal and one of them (polyethylene glycol, [29,158]) has even been used at full scale (750 $m^3$) nitrification with immobilized nitrifying sludge [140].

d) Although smaller beads can be produced, until now, most of the large scale immobilization methods yield biocatalyst particles whose diameter hardly goes under the 1.5 mm (see e.g. [297]. As comparison, the basalt particles used in the reports of Tijhuis [96,143] for nitrification in a three-phase air-lift reactor have diameters of about 0.3-0.5 mm. Although these diameters may double as a logical outcome of biofilm formation, the use of smaller carriers result in much higher specific surface areas and thus overall larger surface for mass-transfer in the reactor. As a consequence, biofilm reactors with such particles show typically higher degradation rates than those for a system based on the same volumetric fraction of artificially immobilized cells (see e.g. Tijhuis et al [96] and Hunik et al.[23]).

This brief analysis shows that most drawbacks of artificially immobilized-cell systems with respect to biofilm-based processes are in fact only of temporary nature. That is to say that the mentioned disadvantages, though important, are more related to an as yet gap in technological advances rather than to real, intrinsic differences between both systems. Indeed, it is expected that, if the recent trends on the development of new materials and immobilization techniques set forth, within a limited period of time suitable biocatalysts produced in an efficient and cost-effective way will be found [24,288].

Yet, it remains the question of why complicating things if they seem simple? That is, why use artificially immobilized cells if biofilms seem to do well? There may be several reasons for that:

a) Biofilms are undefined. Despite the numerous studies on aspects such as formation and growth of biofilms [38, 39,96,143], biofilm structure [224,298-305], detachment of biomass [96,289,290], dynamics of the populations involved [152,196,307] and scale-up [16,127], the mechanisms underlying biofilm processes are not yet well understood. Besides, the knowledge available is, unto a large extent, empirical rather than fundamental. For this reason, biofilm processes are controllable only to a certain extent and tend to be system's bonded rather than generally applicable.

b) As a result of this undefined nature and consequent lack of fundamental knowledge, design, optimization and control of biofilm systems becomes more troublesome and limited than that for their artificially-immobilized counterparts. For instance, the development and use of mechanistic models describing nitrogen removal processes by biofilms is more complex and unreliable because these processes depend, often in an unknown way, of factors such as the composition of the wastewater itself (which determine the bacterial species that will attach), or of the resulting shear stresses acting on the biofilms (which ultimately determine biofilm thickness and density).

c) Since biofilms form by natural attachment of biomass, the start-up phase of biofilm reactors for nitrogen removal (specially for nitrification) tend to be much longer than that for artificially immobilized cells (compare e.g. the results of Hunik et al [23] with those of Tijhuis et al. [96]). Likewise, biofilm reactors tend to react slower than those with entrapped cells to sudden changes in the operating conditions such as influent concentrations or dilution rates (compare e.g. Hunik et al. [23] with van Benthum et al [152]). With this respect, biofilm reactors may have additional problems if the substrate load falls below a given critical value under which biomass detach faster that it grows. Under these circumstances the biofilms may loose a large fraction of their biomass which in turn can result in the collapsing of the reactor as a whole (see also [152, 170]). On the contrary, reactors with artificially immobilized

cells will, under the same circumstances, remain active and viable for much longer because the biomass just stays where it is (except that part that may decay, see [306]). In other words, in artificially immobilized-cell systems the biomass has more "buffer" capacity to face disturbances. This obviously makes the whole system much more stable.

d) Entrapment of biomass in polymeric beads allows selective immobilization. Because of that, it is possible to integrate nitrification and denitrification within a single biocatalyst particle (see sections *Magic-bead and related concepts* and *Autotrophic Ammonia and Nitrite Oxidation*). The potential of biocatalyst particles is thereby fully exploited and does not rely only on the outermost layers of it. Furthermore, the possibility of immobilizing selectively cell populations confer not only important competitive advantages to the relevant populations (for instance, to immobilize only ammonia oxidizers avoiding nitrite oxidizers such as in the Magic-beads), but also much flexibility to a given system because it can be easily adapted to the treatment needs of different streams. This flexibility goes much beyond nitrogen removal alone and can be, in principle, applied to a wide variety of biodegradation processes (see also Martins dos Santos [174,307] and references therein). Biofilm systems, on the contrary, depend on the capacity of cells to attach (which not all can). Any attempt to adapt a given biofilm system to different conditions requires thus the establishment of either different populations or shifts in the proportions of those already present.

All these positive and negative aspects of one type of system with respect to the other should be carefully weighed and evaluated before a clear choice between any of the two types of systems can be made for possible application at full scale. Several feasibility studies for the application of immobilized-cell systems to nitrogen removal have shown that both entrapped-cell systems [148, 26,140,24] and advanced biofilm processes [62,147,148] perform better, cleaner and cheaper than conventional biofilm and activated-sludge systems.

**Modeling nitrogen removal processes**

From the above comparative analysis of the different nitrogen removal systems available or in development, the bulk of the most effective configurations are, in one way or another, based on immobilized-cell technologies. Despite the intrinsic differences between the systems involved, a common aspect is the introduction of a third phase (besides the liquid and gas phases) consisting of the immobilized biomass and its solid support.

This third phase, compared to suspended-cell systems, introduces additional steps for the transport of substrates and products retarding the availability of these to the cells (see Figure 30).

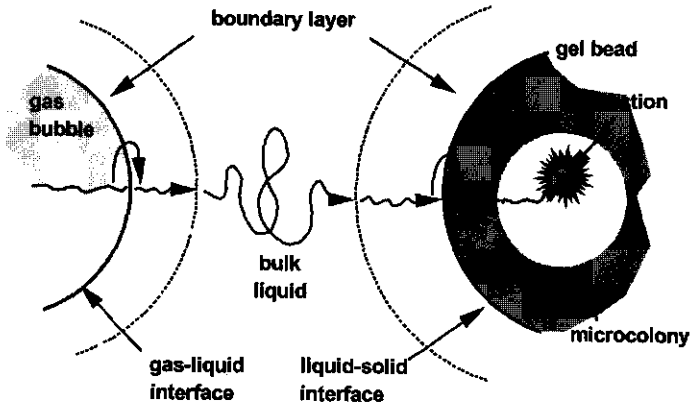


Figure 30 - Transport of substrate from gas phase to immobilized cells. Reproduced from Bailey & Ollis [308] by permission of McGraw Hill, USA.

For instance, gaseous substrates or products (e.g. oxygen or nitrogen, respectively) should diffuse through a boundary layer around the gas bubble to or from the bulk, respectively, and in the bulk they should be transported to (from) the vicinity of the beads. Once there, they must go (come) through an extra boundary layer surrounding the carriers and then diffuse further through layers of biomass and throughout the support matrix before cells can use them. As a result of these steps, the substrates are not immediately available to the cells, and thus substrate-concentration gradients will establish between the particles and the bulk, and across the biocatalysts themselves. Consequently, consumption and growth-rate gradients develop throughout the particles, which in turn leads to the development of biomass profiles across the beads or biofilm layers[45,135,137,309].

In biofilms, additionally, all the factors that determine biofilm formation and maintenance should be accounted for. In the particular case of nitrogen removal several different microorganisms are involved, which means that besides these aspects of transport, growth and activity, the relations among the intervening populations play an essential role in determining the whole nitrogen conversion process in any given reactor system. These kinetic and mass-transfer effects that concern the biocatalyst particle

itself are further determined by the reactor system as a whole, i.e., by macroscopic aspects such as the flow patterns of the gas and liquid phases.

All these interactions make processes based on immobilized cells more complex and troublesome to understand, describe and control than conventional processes based on suspended cells. A way of tackling this complexity and gain insight into the mechanisms involved, is by modeling the interrelationships between the most relevant factors that determine the behavior of a given treatment system. A thorough model soundly validated is an essential tool to design and scale up any nitrogen removal process.

***Mathematical models***

Several models have been developed which describe substrate transport, consumption and growth of immobilized cells both in biofilm and artificially immobilized systems. Initially, most models describing immobilized-cell kinetics were steady-state models, in which the biomass distribution and composition was considered to be both homogeneous throughout the biofilm or bead and constant in time. Also, most of such models consider only zero and first-order kinetics, which gives thus only the upper and lower bounds of microbial conversion. These models give valuable information for design purposes as they reasonably predict nitrogen removal capacities and reactor concentrations at constant input values, but fail to describe transient phenomena such as process start-up or response to changing conditions. In practice, it is very unlikely that all parameters are kept constant, because parameters such as, for instance, influent ammonia or nitrite concentration will vary in time.

From the mechanistic point of view, such models are intrinsically incorrect simply because they assume that biomass does not grow. From a practical standpoint, their most important limitation is that the thickness of the biomass layers should be known, which is in practice often difficult to estimate. Nevertheless, such models were used with reasonable accuracy in several reports to describe nitrification (e.g. 111,112,310,311]), denitrification (e.g. [105] or even integrated nitrification and denitrification (e.g. [312]) under steady-state conditions.

Later, as more became known about the mechanisms underlying immobilized-cell processes, and as numerical techniques (and computers) have improved, several dynamic models were developed in order to describe nitrogen removal in immobilized systems taking into account cell growth and transient conditions (such as influent concentration or temperature/pH changes). In dynamic biofilm models the thickness or density of the biofilm increases or decreases as a function of time according to the environmental conditions (e.g. substrate concentrations or the hydrodynamics)

prevailing locally [190,196, 313]. In dynamic models representing artificially immobilized-cell systems, growth begins from a given (and homogenous) known initial biomass concentration and proceeds in a way that each cell originates into a microcolony that will expand or contract (as result of biomass decay) according to the local substrate concentrations [20-22,295]

Since these models incorporate growth, this means also that growth should be limited (i.e. no infinite biomass allowed) as it obviously is both in real biofilms or beads with immobilized cells. In biofilm models, the biofilm thickness can be restricted by establishing a maximum thickness after which biofilm is lost due to attrition [39,190,314,315]. This maximum, however, is in practice difficult to estimate because it depends not only on the balance between substrate diffusion and consumption but also on the effects that turbulence cause in every particular biofilm. In artificially immobilized cells, a maximum biomass concentration can be set on basis of known maximum cell densities, such in the models of de Gooijer et al.[22], Hunik et al.[23], Martins dos Santos et al. [175] and Monbouquette et al. [316]. Perhaps a more realistic approach was attempted by Wijffels and co-workers [137,139,294], that implemented diffusion limitation within cell colonies as result of colony expansion, and incorporated biomass release as result of colony eruption (see Figure 31). By combining these two mechanisms, they demonstrated that no maximum biomass concentration had to be set.

In addition to the implementation of cell growth and the possibility of simulating transient conditions, some dynamic models were extended so as to include the interrelationships between different cell populations, such as nitrifiers, denitrifiers and heterotrophs in a biofilm or gel bead [23,175,177,190, 287]. This made possible a more rigorous description of the processes involved in general and of integrated nitrification and denitrification in particular. For instance, Wanner & Gujer [189,190] showed mathematically that a stable biofilm is built up of layers with different types of microorganisms.

Thus if nitrifying and denitrifying cells are present, these organisms will compete for oxygen (as denitrifiers are facultative) with the result that nitrifiers will be partially (or totally) overgrown and deprived from oxygen, which in turn affects bioreactor performance. To prevent such an occurrence, Martins dos Santos et al. [175,317] developed a model that successfully described an integrated nitrogen removal system in which denitrifiers are physically forced to remain in the deeper layers (see section *Magic-beads and related concepts*). The predictions yielded by these different models were, in several cases [23,175,194,195,275,317,318]verified experimentally and were found to describe the reality reasonably well.

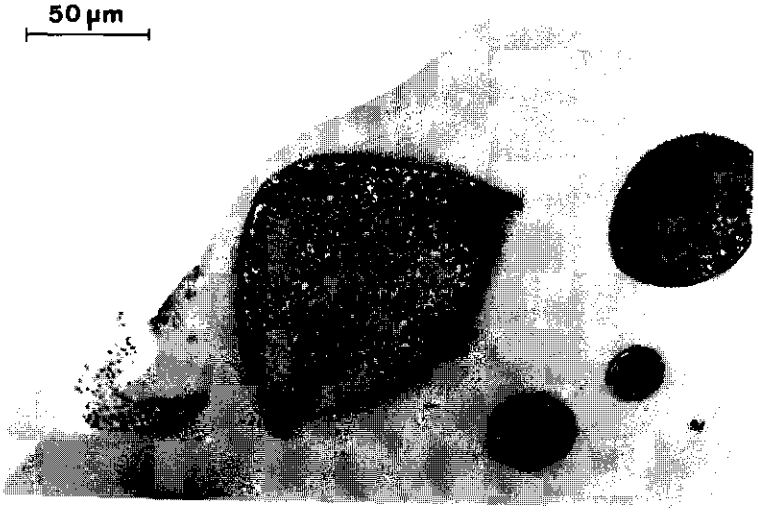


Figure 31 - Eruption of a cell colony at the surface of a bead (reproduced from reference [137] by permission of Springer-Verlag, Germany).

Fore a more extensive description of models describing immobilized cell processes in general, the reader is referred to the works of Salmon [295], Wijffels & Tramper [21], Wijffels et al.[319], Willaert & Baron [20] and Willaert et al. [45].

**Conclusions and future prospects**

The analyses made throughout this paper show that many advanced systems have been developed in the last decade to face the steadily more tight requirements for nitrogen control. The large majority of these processes involve immobilized-cell technology (either naturally attached or artificially immobilized) and tend to be designed in height. This is just the opposite of the treatment plants currently in operation that are based chiefly in activated-sludge systems (suspended biomass in large shallow tanks). Another important tendency is the attempt to integrate nitrification and denitrification within the most compact systems possible. The extreme cases are the systems in which both steps occur within the same biocatalyst particle.

Besides compactness, higher efficiencies are sought as well as cost reduction. Regarding to this, some systems have been proposed in which redundant routes are avoided (such as nitrite into nitrate and again into nitrite) and/or in which nitrogen removal is carried out fully autotrophically. In this way both competition problems between nitrogen removers and other microorganisms are avoided, and the organic matter can be directed to other processes (such as production of biogas or for

intermediary oxidations). It has been also shown that immobilization allows, besides much higher biomass concentrations, a given system to face better variations and extremes (such as low temperatures) in the operating conditions.

Thus far, the most compact systems developed (basically air-lift-like configurations with (co-)immobilized cells) are still operating at lab or pilot scale. Two exceptions, one with attached biomass (Gist-Brocades in The Netherlands) and the other with entrapped cells (Hitachi in Japan), have been reported to function well at scales of 300 and 750 $m^3$, respectively. Several technical-economical studies have shown that such processes may substitute with considerable advantages the more conventional ones.

Insight into the mechanisms underlying many of these processes has been given by fundamental research on the different microbiological and engineering aspects involved, and by the development and validation of suitable models describing their behavior under a wide range of conditions.

**Acknowledgments**

We thank W. van Benthum of the Technical University Delft, The Netherlands, for disclosing part of his results prior formal publication. A grant from the Portuguese National Scientific Board (JNICT) is also gratefully acknowledged (BD/3753/94).

**References**

1. Robertson LA & Kuenen JG. Nitrogen removal from water and waste. Fry, G.M, Herbert, R, Jone, C., Watson-Craik, I. (Eds) In: Microbial control of pollution. 1992, Society for General Micropbiology, Cambridge University Press
2. Zevenboom, W, Rademaker, M, Colijn, F. Exceptional algal blooms in Dutch sea waters: North Sea pollution and technical strategies for improvement. IAWPRC/EWPCA/NVA, 1990, Amsterdam, The Netherlands, 473-486
3. Abeliovich, A. Transformations of ammonia and the environmental impact of nitrifying bacteria. Biodegradation. 1993; 3:255-264
4. Baumgartner M, Samelunck, F, Bock E. Conrad, R. Production of nitric oxide by ammonium-oxidizing bacteria colonizing building stone. FEMS Microb. Ecology, 1991, 85:95-100
5. Crutzen PJ, Andreae MO. Biomass burning in the tropics; imppct on atmospheric chemistry and biogeochemical cycles. Science, 1990; 250:1669-1678
6. Mateju V, Cizinska S, Krejci J, Janoch T. 1992. Biological water denitrification: a review. Enzyme Microbial Technology. 1992; 14:170183.
7. Hiscock KM, Lloyd JW, Lerner DN. Review of natural and artificial denitrification of groundwater. Wat. Res. 1991; 25:1099-1111
8. Wang WC, Yung YL, Lacis AA, T., Hansen, J.E. 1976. Greenhouse effects due to man-made perturbations of trace gases. Science 1976; 194:685-690

9. Rasmussen RA, Khalil MAK. Atmosferic trace gases: trends and distribution over the last decade. Science, 1986, 232:1623-1624.

10. Cole JA. Controlling environmental nitrogen through microbial metabolism. Trends in Biotechnology, 1993, 11:368-372

11. Rowland FS, Isaksen I.S.A., (eds.). The changing atmosphere. 1988, Jonh Wiley & Sons, New York, USA:

12. Council of the European Communities Official Journal. EC urban wastewater treatment directive nr. 91/271, 135/140, 30 May 1991

13. Sayre, IMJ., 1988. Internation standards in drinking water. J. Am. Water. Works Ass. 80:53-60

14. Tanaka K Emori, H. Development of biological nutrient control in Japan. The 2nd Japan-The Netherlands Workshop on Integrated Water Quality Management, 1993

15. Heijnen JJ, van Loosdrecht MCM, Mulder R, Weltevrede R, Mulder A. Large scale anaerobic-aerobic treatment of complex industrial wastewater using biofilm reactors. Wat. Sci. Technol. 1991; 23:1427-1436

16. Heijnen JJ, van Loosdrecht MCM, Mulder R, Weltevrede R, Mulder A. Development and scale-up of an aerobic biofilm air-lift suspension reactor. Wat. Sci. Technol. 1993; 27:253-261

17. Tijhuis L. The biofilm airlift suspension reactor: biofilm formation, detachment and heterogeneity. 1994, PhD thesis, Delft University of Technology, Delft, The netherlands.

18. Lazarova V, Manem J. Advances in Biofilm aerobic reactors ensuring effective biofilm activity control.. 1994; 29(10-11):;319-327

19. Karel SF, Robertson CR. Autoradiographic determination of mass transfer limitations in immobilized cell reactors. Biotechnol. Bioeng. 1989; 34:320-336

20. Willaert RG, Baron GV. Gel entrapment and microencapsulation: Methods, applications and engineering principles. Rev. Chem. Eng. 1996, 1-2:1-161

21. Wijffels RH and Tramper J. Nitrification by immobilized cells. Enz. Microb. Technol., 1995; 17:482-492

22. de Gooijer CD, Wijffels RH, Tramper J. Growth and substrate consumption of Nitrobacter agilis cells immobilized in carrageenan: part 1. Dynamic modeling. Biotechnol. and Bioeng. 1991; 38:224-231.

23. Hunik JH, Bos CG, Van Den Hoogen P, De Gooijer CD., Tramper J. Co-immobilized *Nitrosomonas euroapaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in k-carrageenan gel beads. Biotechnol. Bioeng. 1994; 43:1153-1163.

24. Leenen EJMT, van Boxtel AMGA, Englund G, Tramper J., Wijffels RH. Reduced temperature sensitivity of immobilized *Nitrobacter agilis* caused by diffusion limitation. Enz. Microbial Technol. 1997, in press

25. Hunik JH, Tramper J and Wijffels RH. A strategy to scale-up nitrification processes with immobilized nitrifying cells. Bioprocess Eng. 1994b, 11:73-82.
26. Chudoba P, Pujol R, Emori H, Bourdelot JC, Rovel JM. Comparison of a system using immobilized microorganisms with a conventional activated sludge process for wastewater treatment. In: Immobilized cells: basics and applications (Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J, Eds). Elsevier Science, The Netherlands, 1996, pp. 710-717
27. Rusten B , Hem LJ, Odegaard H. Nitrogen removal from dilute wastwater in cold climate using moving-bed biofilm reactors. Wat. Environm. Res., 1995, 67:65-86
28. Frijters CTMJ, Eikelboom DH, Mulder A, Mulder R. Treatment of municipal wastewater in a CIRCOX® airlift reactor with integrated denitrification. Proceedings of the 3rd International IAQW special conference on biofilm systems, Copenhagen, 27-30 August 1996
29. Tanaka K, Sumino T, Nakamura H, Ogasawara T, Emori H. Application of nitrification by cells immobilized in polyethylene glycol. In: Immobilized cells: basics and applications (Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J, Eds). Elsevier Science, The Netherlands, 1996, pp. 718-724
30. Tsai Y-L, Schlasner SM, Tuovinene OH. Inhibitor evaluation with immobilized *Nitrobacter agilis* cells. Appl. Environm. Microbiol. 1986, 52:1231-1235
31. Keweloh H, Heipieper H_J, Rehm H-J. Protection of bacteria against toxycity of phenol by immobilization in calcium alginate. Appl. Microbiol. Biotechnol. 1989; 31:383-389.
32. Sikkema J, de Bont, J, Poolman, B. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Reviews. 1995, 59:2:201-222.
33. Hunik JH, Tramper J, Meijer HG. Kinetics of *Nitrosomonas europaea* at extreme susbtrate, product and salt concentrations.Appl. Microbiol. Biotechnol. 1992, 37:802-807
34. Heijnen JJ. Biological industrial wastewater treatment minimizing biomass production and maximizing biomass concentration. Ph.D. thesis, Delft University of Technology, Delft, The Netherlands
35. Van Loosdrecht MCM, Heijnen JJ. Bioreactors for wastewater treatment. Trends in Biotechnol. 1993; 11:117-121
36. Heijnen JJ, van Loosdrecht MCM, Mulder A., Tijhuis, L. Formation of biofilms in a biofilm air-lift suspension reactor. Wat. Sci. Technol., 1992; 26:647-654
37. Characklis WG, Wilderer P.- A. (Eds.) 1989. Structure & function of biofilms. John Wiley & Sons, New York, USA:
38. Characklis WG, Marshall, KC. (Eds.) 1990. Biofilms. John Wiley & Sons, New York, USA:
39. Bryers J.D. Modeling biofilm accumulation. In: Bazin, MJ, Prosser, JI. (eds) Physiological models in microbiology, 1988, vol. 2, CRC press, Boca Raton, FL, USA, pp109-144.
40. Van Loosdrecht MCM. Bacterial adhesion. PhD Thesis, Wageningen Agricultural University, The Netherlands, 1988

41. Van Loosdrecht MCM, Heijnen, JJ. Biofilm processes. In: Immobilized living cell systems. Willaert, R.G., Baron, G.V., de Backer, L. (Eds.), 1996, pp 255-271, John Wiley & Sons, New York, USA

42. Wang B. Nitrogen removal by a submerged biofilm process with fibrous carries. Wat. Sci. Technol. 1992, 26:2039

43. Melo L, Bott RT, Fletcher M, and Capdeville B. (Eds.), Nato Series, 1992, pp. 449-453.

44. Bryers JD, Characklis WG. 1982. Biofilm accumulation and activity.: na process design approach. In: Biofilms - Science and Technology. Melo, LF, TR Bott, M Fletcher and B Capdeville 1992, (Eds.), L.F., Nato Series, Kluwer Academic publishers, Doordrecht, The Netherlands. 1992, 221-239

45. Willaert RG, Baron GV, de Backer L. Immobilized living cell systems. 1996, John Wiley & Sons, New York, USA

46. Scott CD. Immobilized cells: a review of recent literature. Enz. Microb. Technol., 1987, 9:66-73

47. Klein J, Vorlop K-D. Immobilization techniques. In: Comprehensive Biotechnology, Moo-Young, M, Cooney CL, Humphrey AE., (Eds.) Pergamon press, Oxford, Uk, 1985; 2:203-224.

48. Hulst AC, Tramper J, van 't Riet K, Westerbeek JMM: A new technique for the production of immobilized biocatalyst in large quantities. Biotechnol. Bioeng. 1985; 27:870-876

49. Metcalf & Eddy Inc. Wastewater engineering: treatment disposal reuse. 2nd De. McGraw-Hill, New York, USA, 1979.

50. Chen JM, Lim BS, Al-Ghusain IA, Hao O, Lin C, Davis P, Kim M, Huang J. Biological fixed-film processes. Wat. Environm. Res. 1993; 65:336-344

51. Capdeville B, Rols JL. Introduction to biuofilms in water and waste water treatment. In: Biofilms - Science and Technology. Melo, L.F., T.R. Bott, M. Fletcher and B. Capdeville 1992, (Eds.), L.F., Nato Series, Kluwer Academic publishers, Doordrecht, The Netherlands. 1992, 13-20

52. Garrido JM. Nitrificación e desnitrificación de aguas residuais con concentracións elevadas de formaldehido e urea. PhD thesis 1996, Univ. de Santiago de Compostela, Spain

53. Bishop PL, Kinner NE. Aerobic fixed-film processes. In: Biotechnology, 1986, Vol. 8, Ed. VCH, Weinheim, Germany

54. Andersson B, Asprengen H, Parker DS, Lutz MP. High rate nitrifying trickling filters. Wat. Sci. Technol. 1994; 29(10-11):47-52

55. Mendez R, Lema JM. Biofilm reactors technology in wastewater treatment. In: Melo et al., (eds) Bioofilms - Science and Technology, 1992 NATO ASI series, Kluwer Academic Publishers, Doordrecht, The Netherlands.

56. Dalsgaard T, de Zwart J, Robertson LA, Kuenen JG, Revsbech NP. Nitrification, denitrification and growth in artificial *Thiosphaera pantothropha* biofilms as measured with a combined microsensor for oxygen and nitrous oxide. FEMS Microbiology Ecology, 1995; 17:137-148.

57. Nurizzo C, Mezzanotte V. Groundwater biodenitrification on sand fixed film reactor using sugars as organic carbon source. Wat. Sci. Technol. 1992; 26(3-4):827-834

58. Sagberg P, Dauthille P, Hamon M. Biofilm reactors: a compact solution for the upgrading of wastewater treatment plants. Wat. Sci. Technol. 1992, 26:733-742

59. Watanabe S, Okabe Y, Hirata K, Masuda S, Ishiguro M. Simultaneous removal of organic materials and nitrogen in micro-aerobic films. Wat. Sci. Technol. 1995; 31:195-203

60. STOWA, Handboek stikstof verwijdering. 1993b, STOWA, Zoetermeer, The Netherlands, verslag 93-07, pp. 43-67

61. STOWA/STORA. Nitrificatie en denitrificatie in compactsystemen:inventarisatie en praktijservaring, STORA, Den Haag, The Netherlands 1992

62. Kruit J, Wiegant WM. Compactsystemen voor de behandeling van stedelijk afvalwater.: een Haalbarheidsstudie. RWZI 2000, den Haag, , Rapport nr 94-10, 1994

63. Pujol R, Hamon M, Kandel X, Lemmel H. Biofilters; flexible, reliable biological reactors. Wat. Sci. Technol. 1994; 29(10-11), 33-38

64. Pujol R, Canler, JP, Iwema I. Biological aerated filters; an attractive and alternative biological process. Wat. Sci. Technol., 1992, , 26(3-4):693-702-38

65. Canler JP, Perrewt JM. Biological aerated filters: assessment of the process based on 12 sewage treatment plants. Wat Sci. Technol. 1994; 29(10-11):13-22

66. Richard Y, Partos J. Elimination biologique des nitrates en vue de la production d'eau potable. Bilann de fonctionnement de deux installations industrielles. T.S.M. L'eau, 1986,3:141-147

67. Carrand G, Capon B, Rasconi A, Brenner R. Elimination of carbonaceous and nitrogenous pollutants by a twin-stage fixed process. Wat. Sci. Technol. 1990; 22:261-272

68. Jimenez B, Capdeville B, Roques H, Faud GM. Desigh considerations for a nitrification-denitrification process using two fixed-bed reactors in series. Wat. Sci. Technol., 1987; 19:139-150

69. Çeçen F, Gönenç IE. Criteria for nitrification and denitrification of high-strength wastees in two upflow submerged filters. Wat. Environm. Res. 1995; 67:132-142

70. Rogalla F, Bourbigot MM. New developments in complete nitrogen removal with biological aerated filters. Wat. Sci. Technol. 1990, 22:273-279

71. Meaney B. Strickland ET. Operating experiences with submerged filters for nitrification and denitrification. Wat. Sci. Technol. 1994, 29:119-126

72. Daamen, E.J., Delgorge, F.H., van Loosdrecht, M.C.M., Heijnen, JJ:: The biogenerator: a new biofilm concept without backwash requirements. Proceedings of the 3rd International IAQW special conference on biofilm systems, Copenhagen, 27-30 August 1996

73. Mann A, Mendoza-Espinoza LG, Stephenson T. A comparison of pilot-scale biological aerated filters using sunken and floating media. Proc. of the 3rd Specialized Conf. On Biofilm Systems, Copenhagen, Denmark, 1996.

74. Ødegaard H, Rusten B, Westrum T. A new moving-bed biofilm reactor - applications and results. Wat.Sci. Technol. 1994; 29:157-167

75. Lessel TH, Upgrading and nitrification by submerged biofilm reactors - experiences froam a large scale plant. Wate. Sci. Technol. 1994; 29:167-174

76. Eikelboom DH, Bral EA. Linpor-sponges als dragermaterial bij de aerobe zuivering van rioolwater. Rapport RWZI-2000 90-07, DBW/RIZA, Lelystad, 1990

77. Pastorelli G, Andreottola G, Canziani R, Frangipane E, de Pascalis F, Gurrieri G, Rozzi, A. Pilot plant experiences with moving bed biofilm reactors. Proc. of the 3rd Specialized Conf. On Biofilm Systems, Copenhagen, Denmark, 1996.

78. Hem L. Rusten B, Odegaard H. Nitrification in a moving bed biofilm reactor. Wat. Res. 1994; 28:1425-1433

79. Reinmann H. The LINPOR-process with fixed biomass for on plastic foam - practical aspects and results. In: La biomasse fixeé dans le traitement des eaux. 37es Journées Int. CEBEDEAU, Liège, 23-25 May, pp 353-361

80. Rusten B, Siljudalen JG, Nordeidet B. Upgrading nitrogen removal with the KMT moving bed biofilm process. Wat. Sci. Technol., 1994,29:185-195

81. Pujol R. (Ed.) Proceedings of the 1st IAWQ International Specialized Conference on Microorganisms in activated sludge and biofilm processes. Wat Sci. Technol. , 1994, 29(7), 395pp

82. Rogalla F, Harremoes P. (Eds.) Proceedings of the 2st IAWQ International Specialized Conference on Biofilm Reactors. Wat Sci. Technol. , 1994, 29(10-11), 545pp

83. Van Loosdrecht MCM., Tijhuis L. (Eds). Proceedings of the IAWQ International Specialized Conference on Biofilm Structure, Growth and Dynamics. Wat Sci. Technol. , 1995, 32(8), 290pp

84. Arvin E, Harremoes P. (Eds.) Proceedings of the 1st IAWQ International Specialized Conference on Biofilm Reactors. Wat Sci. Technol. , 1989; 26

85. Harremoes P. Proc. of the 3rd Specialized Conf. On Biofilm Systems, Copenhagen, Denamrk, 1996

86. Henze M, Harremoes P. Anaerobic treatment of watewater in fixed film reactoers,- a literature review. Wat. Sci. Technol. 1983, 15:1-101

87. Eggers, E. Nitrification and denitrification in compact systems. Second Japanese-Dutch Workshop on Integrated water quality management. October 20-23, 1993, Myiazaki, Japan

88. STOWA, Nitrificatie en denitrificatie in compactsystemen: evaluatie praktijk onderzoek. 1993a, Stowa, Zoetermeer, The Netherlands, Rapport nr 93-13

89. Christensen FR., Holm. KG., la Cour Jansen, J. Biofilm structure - an important and neglected parameter in waste water treatment. Wat. Sci. Technol. 1989; 21:805-814

90. Grady CPL, Lim HC. Biological wastewater treatment; theory and applications. Marcel Dekker, Inc. New York, 1980, 850 pp

91. EPA, U.S. Environmental Protection Agency. 1975 Process design manual for nitrogen control. USA.
92. Winkler M. Biological treatment of wastewater. Hollis-Horwood, Chichester, USA, 1981
93. Barnes D, Bliss PJ. Biological control of nitrogen in wastewater treatment. 1983, E & F. Spon. Ltd. London, 146 pp.
94. STOWA, Behandeling van stikstofrijk retourstommen op rioolwaterzuiveringsinrichtingen. Praktijkonderzoek aan membraan-bioreactoren bij het slibverwerkingsbedrijf Sluiesjesdijk. 1995, STOWA, The Netherlands
95. Sutton PM, Mishra PN, Crawford PM. Combining biological and physical processes for complete treatment of oily wastewaters. Int. Biodeter. Biodeg., 1994, 33:3-21
96. Tijhuis L, Huisman JL, Hekkelman HD, van Loosdrecht MCM, Heijnen JJ. Formation of nitrifying biofilms on small suspended particles in air-lift reactors. Biotechnol. Bioeng. 1995; 47:585-595
97. Jeris JS, Owens RW, Hickey R. Biological fluidized bed treatment for BOD and nitrogen removal. J. Wat. Poll. Control Fed. 1977; 49:816-821
98. Cooper PF. The use of biological fluidized beds for the treatment of domestic and industrial wastewater. The Chem Eng., 1981; 8/9:373-376
99. Sutton PM, Mishra PN. Fluidized bed biological treatment: effects of scale-up on system performance. Wat Sci. Technol., 1990; 22:419-430
100. MacDonald DV. Denitrification by fluidized biofilm reactor. Wat. Sci. Technol., 1990; 22:451-462
101. Hare RW, Sutton PM, Mishra PN, Potochnik KF. Utilization of fluidized bed biological treatment at General Motors facilities: pilot and full scale results. Proceedings of the Industrial Waste Symposia, 1988, Wat. Poll. Control Fed., Dallas, Usa
102. Nutt SG, .Melcer H. and Pries JH. Two-stage biological fluidized bed treatment of coke-plant wastewater for nitrogen control. J. Wat. Poll. Control Fed., 1984; 56:851-863
103. Jördening H-J., Zastrutzki M, Feuerhake E. High rate nitrification-denitrification with immobilized systems. Poster presented at the 6th European Congress on Biotechnology, Florence, Italy, June 13-17, 1993
104. Croll BT, Hayes CR. Environm, Pollut. 1988; 50:163-167
105. Coelhoso I, Boaventura R, Rodrigues, A. Biofilm reactors: an experimental and modeling study of wastewater denitrification in fluidized-bed reactors of activated-carbon particles. Biotechnol. Bioeng. 1992; 40:625-633
106. Vredenbergt L, Nielsen K, Potma A, Holm H, Kristensen D and Sund C. Fluid bed biological nitrification and denitrification in high salinity wastewater. Proceedings of the 3rd International IAQW special conference on biofilm systems, Copenhagen, 27-30 August 1996
107. Cooper PF, Wheeldon DH. Complete treatment of sewage in a two-stage fluidized bed system Part 1. Wat. Poll. Control. 1982; 447-464

108. Popel HJ, Kristeller W.: Post-denitrification at the Frankfurt-Niederrad wastewater treatment plant by fluidized-bed technology. Proceedings of Aquatech 96- Advanced wastewater treatment, Amsterdam, 23-25 September 1996, pp. 154-162

109. Narjari NK, Khilar KC, Mahajan SP. Biological denitrification in a fluidized bed. Biotechnol. Bioeng. 1984; 26:1445-1448

110. Nilsson I, Ohlson S. Columnar denitrification of water by immobilized cells. Appl Microbiol Biotechnol. 1982; 14: 86-90

111. Tanaka H, Uzman S, Dunn IJ. Kinetics of nitrification using a fluidized bed sand reactor with attached-growth. Biotechnol. Bioeng. 1981, 21:423-445

112. Denac M, Uzman S, Tanaka H, Dunn IJ. Modeling of experiments on biofilm penetration effects in a fluidized-bed nitrification reactor. Biotechnol. Bioneng. 1983, XXV:1841-1861

113. Cooper PF, Williams SC. High-rate nitrification ina biological fluidized bed. Wat. Sci. Technol. 1990; 22:431-442

114. Rittmann BE. Aerobic biological treatment. Environm. Sci. Technol., 1987; 21-128-136

115. Kurt M, Dunn IJ, Bourne J R. Biotechnol. Biological denitrification of drinking water using autotrophic organims with hydrogen in a fluidized-bed biofilm reactor. Bioeng. 1987; 24:493-501

116. Lazarova V, Capdeville B and Nikolov L. Biofilm performance of a fluidized bed biofilm reactor for drinking water denitrification. Wat. Sci. Technol. 1992; 26:555-566

117. Holló J, Czako L. Nitrate removal from drinking water in a fluidized bed biofilm reactor for denitrification in a bioreactor. Acta Biotechnol. 1987; 7: 417-423

118. Hancz P, Csikor Zs, Miháltz P, Czakó L. High performance biodenitrification system for industrial use. Proceedings of 10th Symposium for Applied Biotechnology, Gent, Belgium, 26-27 September 1996

119. Fernandez - Polanco F, Real FJ, Garcia PA. Behaviour of an anaerobic/aerobic pilot scale fluidized bed for the simultaneous removal of carbon and nitrogen. Wat. Sci. Technol. 1994; 29:339-346

120. Acítores M, Garcia PA, Fernandez-Polanco F. Behaviour of a fluidized bed for the simultaneous organic carbon and nitrogen removal in domestic sewage. International Symposium on Environmental Biotechnology, Oostend, Belgium, pp. 235-238

121. Ruggeri B, Caire G, Spechia V, Sassi G, Bosco F and Gianetto, A. Determination of optimal biofilm activity in a biological fluidized bed (BFB) reactor. Wat Sci. Technol. 1994; 29:347-352

122. Imai T, Kusuda T, Furumai H. Kinetic study and mathematical modelling of biofilm in an anaerobic fluidized bed. Wat. Sci. Technol. 1994; 29:369-376

123. Ozturk I, Turan M, Idris H. Scale up and biomass hold-up characteristics of fluidized bed reactors. Wat. Sci. Technol. 1994; 29:353-360

124. Chisti MY. Airlift biorreactors. 1989. Elsevier Applied Science, Essex, , UK.

125. Tang W-T, Wisecarver K, Fan L-S. Dynamics of a draft tube gas-liquid-solid fluidized bed bioreactor for phenol biodegradation. Chem. Eng. Sci. 1987; 42:2123-2134

126. Fan LS, Fujie K., Long TR. Some remarks on gas-liquid mass transfer and biologicakl phenol degradation in a draft tube gas-liquid-solid fluidized bed bioreactor. A.I.Ch.E. Symp. Series, 1984;80:102-109

127. Heijnen JJ. Scale-up of immobilized cell reactors. In: Immobilized cells: basics and applications (Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J, Eds). Elsevier Science, The Netherlands, 1996, pp. 497-504

128. Bennemann H, Feldmann M, Hempel DC. Nitrifikation mit immobilsierten Bakterien. WasserAbwasser. 1991;132 686-689

129. Hano T, Matsumoto M, Kuribay K and Hatate Y.: Biological nitrogen removal in a bubble column with a draught tube. Chemical Engineering Science 1992, 47:3737-3744.

130. Nilsson I, Ohlson S, Haggstrom L, Molin N, Mosbach K (1980). Denitrification of water using immobilized *Pseudomonas denitrificans* cells. Eur. J. Appl Microbiol Biotechnol. 10: 261--274

131. Van Ginkel CG, Tramper J, Luyben KChAM, Klapwijk A. Characterization of *Nitrosomonas europaea* immobilized in calcium alginate. Enz. Microb. Technol. 1983; 5:297-303

132. Tramper J. Nitrification and denitrification by immobilized bacteria, Proc. 3rd European Congress on Biotechnology, VCH, 1984; IV: 363—368

133. Tramper J Nitrification and denitrification by immobilized viable cells. In: *Enzyme Engineering 8*, ed. A.I. Laskin, L. Mosbach, D. Thomas, L.B. Wingard Annuals of the New York Academy of Sciences. 1987; 501: 362—366

134. Wijffels RH. and J. Tramper. Performance of growing *Nitrosomonas europaea* cells immobilized in carrageenan. Appl. Microbiol. Biotechnol. 1989, 32: 108-112.

135. Wijffels RH, De Gooijer CD, Kortekaas S, Tramper J. Growth and substrate consumption of *Nitrobacter* cells immobilized in κ-carrageenan. Part 2: Model evaluation. Biotechnol. Bioeng. 1991; 38: 232-240

136. Wijffels RH, De Gooijer CD, Scheppers AW, Beuling EE, Mallée LF., Tramper J. Dynamic modeling of immobilized *Nitrosomonas europaea*: implementation of diffusion limitation over expandig micro-colonies. Enz. Microbial., Technol..1995; 17: 462-471

137. Wijffels RH, Schepers AW, Smit M, De Gooijer CD, Tramper J. Effect of initial biomass concentration on the growth of immobilized *nitrosomonas europaea*. Appl. Microbiol. Biotechnol. 1994; 42:153-158

138. Wijffels RH, De Gooijer CD, JM, Tramper J. Gel immobilized living-cell systems: Part 2. In: Immobilized living cell systems. Willaert, R.G., Baron, G.V., de Backer, L. (Eds.), 1996, John Wiley & Sons, New York, USA, 215-236

139. Wijffels RH, Schukking A, and J. Tramper. Characterization of a denitrifying bacterium immobilized in k-carrageenan, Applied Microbiol. Biotechnol. 1990, 34:399-403

140. Emori H, Mikawa K, Hamaya M, Yamaguchi T, Tanaka K, Takeshima T. PEGASUS, innovative biological nitrogen removal process using entrapped nitrifiers. In:

Immobilized cells: basics and applications (Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J, Eds). Elsevier Science, The Netherlands, 1996, pp. 546-555

141. Wilke Th, Vorlop K-D. Nitrification in PVAL beads: influence of pH and temperature on nitrite oxidation. In: Immobilized cells: basics and applications (Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J, Eds). Elsevier Science, The Netherlands, 1996, pp. 718-724
142. Hunik J.H., Bos C.G., Van Den Hoogen P., de Boer, W.,. Smit, M.., Tramper J. Quantitative determination of the spatial distribution of *Nitrosomonas euroapaea* and *Nitrobacter agilis* cells co-immobilized in κ-carrageenan gel beads by a fluorescent-antibody labelling technique. Appl. Environm. Microbiol. 1993, 9:1951-11954
143. Tijhuis L, van Loosdrecht MCM., Heijnen J.J. Formation and growth of heterotrophic aerobic biofilms on small suspended particles in air-lift reactors. Biotechnol. Bioeng. 1994, 44:595-608.
144. Tada M., Kimata, T. Mori, N., Emori, H. Nitrogen removal systems using immobilized microorganisms in synthetic resin. Hitachi Review, 1990; 39, 376-386.
145. Jian-an C, Nieuwstad Th. Modelling the three phase flow in a pilot-scale internal-loop airlift reactor for wastewater treatment. Environm. Tech. 19992; 13: 101-113
146. Witteveen-Bos, raadgevende ingenieurs. Application of the three-phase airlift reactor for the purification of municipal wastewater.: technical and economical evaluation. 1990 (as referred by Tihuis, 1994)
147. Kampf R, de Graaf TP, Mulder A. Technological-economical evaluation of the application of a three-phase airlift loop reactor for the municipal treatment of wastewater. TNO Research report n° R90/421. 1990, TNO, Delft, The Netherlands
148. Witteveen-Bos, raadgevende ingenieurs, Technisch-economische verkenning van zuivering met geimmobiliseerde nitrificerende bacterien, 1991, Lls.144.1
149. Leenen EJMT, Tramper J., Wijffels RH. Nitrification with artificially immobilized cells: model and practical system! Submitted.
150. Hao X, Nieuwstad Th J. Feasibility of denitrification in air-lift loop reactors. Environmental Technology 1994; 15:155-163.
151. Badot R, Coulom T, de Longeaux N, Badard M, Sibony J. A fluidized bed reactor: the BIOLIFT process. Wat. Sci. Technol. 1994; 29:329-338
152. Van Benthum WAJ, van Loosdrecht MCM, Heijnen JJ. Nitrogen removal using nitrifying biofilms and denitrifying suspended growth in a biofilm air-lift suspension reactor. Proceedings of the 3rd International IAQW special conference on biofilm systems, Copenhagen, 27-30 August 1996
153. Matsui T, Kyosai S, Takahashi M. Progress in the research on application of biotechnology to wastewater treatment. Proceedings of the 4th WPCF/JSWA joint technical seminar on sewage treatment technology. May 17-19, 1990, Tokyo, Japan
154. Czikor Zs, Czakó L, Miháltz P, Holló J. Fluidization characteristics and factors affecting oxygen diffusinal limitation in mixed cultures of nitrifiers. Abstract paper for the IAWPRC 94 conference.

155. Kossen NWF. Scale-up in Biotechnology. In: Varder-Sukern, I, Sukern, S.S. (eds). Recent Advances in Biotechnology. Kluwer Academic Publishers, Amsterdam, The Netherlands, 1992; 101-131

156. Verlaan P. Modelling and characterization of an air-lift loop bioreactor. PhD. Thesis, Wageningen Agricultural University, The Netherlands, 1987; 107-120

157. Eggers E, Schellinkhout A, van der Schot FJ. Deep-shaft systemen: een inventarisatie. Lelystad, The Netherlands: RWZI 2000, 91-02, 1991

158. Tanaka K, Tada M, Kimata T, Fujii Y, Mizuguchi T., Mori N, Emori H. Development of a new nitrogen removal system using nitrifying bacteria immobilized in synthetic resin pellets. Water Science and Technology. 1991; 23:681-690.

159. Tanaka K. Recent advances in biological nutrient control in Japan. 5th WEF/JSWA joint Technical seminar on sewage treatment technology. 1992

160. Takeshima T, Motegi K, Emori H, Nakamura H. "Pegasus", an innovative high-rate BOD and nitrogen removal process for municipal wastewater. 66th W.E.F. Annual Conference, 1993.

161. Ludzack FJ and Ettinger MB. Controlling operation to minimize activated sludge effluent nitrogen. JWPCF 1962; 34:920-931.

162. Taniguchi N, Koike S, Murakami T. and Nakayama S. High efficiency nitrogen removal using multi-stage air-lift recirculation for nitrogen and denitrification process. WPCF Anual Conference, October 1988, Dallas, Texas, USA

163. de Gooijer CD, Bakker WAM, Beeftink HH and Tramper J. Series of bioreactors: an overview of design procedures and practical applications. Enz. Microbial Technol. In press.

164. Martins dos Santos VAP, Tramper J, Wijffels R.H. Simultaneous nitrification and denitrification using immobilized microorganisms, Biomat. Artif. Cells & Immob. Biotechnol., 1993; 21:317-322.

165. Martins dos Santos VAP, Tramper J, Wijffels RH. Integrated Nitrification and Denitrification with Immobilized Bacteria, in Biofilms - Science and Technology, L.F. Melo, T.R. Bott, M. Fletcher and B. Capdeville (Eds.). 1992 Nato ASI Series, pp. 449-453.

166. De Gooijer CD. Werkwijze voor het uitvoeren van biotechnologische processes in meer-traps loopreactoren. Dutch patent nr 89.01649, 1989.

167. Bakker WAM, Van Can HJL, Tramper J, De Gooijer CD,. Hydrodynamics and mixing in a multiple air-lift loop bioreator. Biotechnol. Bioeng. 1993; 42:994-1001 168. Bakker, W.A.M. Design, characterization and application of the multiple air-lift loop reactor. PhD thesis, Wageningen Agricultural University, Wageningen, The Netherlands, 1995

169. Van Benthum WAJ, van Loosdrecht MCM, Heijnen JJ. Control of heterotrophic layer formation on nitrifying biofilms in a biofilm air-lift suspension reactor. Biotechnol. Bioeng. 1997; 53:397-405

170. Tijhuis L, Dynamics of population and biofilm structure in the biofilm airlift suspension reactor for nitrogen and carbon removal. Wat. Sci. Technol. 1994;29:377-384

171. McCarty P, Beck L, Aman P. Biological denitrification of wastewaters by addition of organic materials. Proc. 24th Ind. Waste Conf. Purdue University, West Lafayette, Indiana, USA, 1969; 1271-1285

172. Van Benthum WAJ, van Loosdrecht MCM. Removal of nitrogen compounds from wastewaters. International issue of the IOP on Environmental Biotechnology, 1995, 10-11 (IOP Milieubiotechnologie, 5:58-59)

173. OTV. The BIOLIFT® process. Company brochure 1996.

174. Martins dos Santos VAP, Tramper J, RH Wijffels. The Magic Bead Concept: an integrated approach for nitrogen removal with co-immobilized microorganisms. Appl. Microbiol. Biotechnol. 1996; 45:447-453.

175. Martins dos Santos VAP, L Marchal, Tramper J, R.H. Wijffels. Modelling and evaluation of an integrated nitrogen removal system with co-immobilized microorganisms. Biotechnol. Prog. 1996; 12:163-171

176. Wijffels RH, Eekhof M, De Beer D, Van den Heuvel JC, Tramper J. Pseudo-steady state oxygen concentration profiles in an agar slab containing growing *Nitrobacter agilis*. J. Fermentation Bioeng. 1995; 79:167-170

177. Tartakovski B, Kotlkar, E, Sheintuch M. Coupled nitrification-denitrification processes in a mixed culture of co-immobilized processes: analysis and experiment. Chem. Eng. Sci., 1996;51:2327-2336

178. Martins dos Santos VAP, Verschuren P, Smit M, van den Heuvel H, Tramper J, Wijffels RH. pH effects on coupled nitrification and denitrification measured by specific microelectrodes. Submitted.

179. Siegrist H, Gujer W. Demonstration of mass transfer and pH effects in a nitrifying biofilm. Water Research, 1987, 21:1481-1487

180. Arvin E, Kristensen GH. Effect of denitrification on the pH in biofilms. Water Sci. Technol. 1982; 14: 833-848.

181. Szwerinski H., Arvin E, Harremoes P. pH decrease in nitrifying biofilms. Water Research, 1986; 20: 971-976

182. Kokufuta E, Shimohashi M, Nakamura I. Simultaneously occurring nitrification and denitrification under oxygen gradient by polyelectrolyte complex-coimmobilized *Nitrosomonas* and *Paracoccus denitrificans* cells. Biotechnol. Bioeng., 1988, 31:382-384.

183. Kokufuta E, Yukishige M, Nakamura I. Coimmobilization of *Nitrosomonas* and *Paracoccus denitrificans* cells polyelectrolyte complex-stabilized calcium alginate gel. J. Ferment. Bioeng. 1987; 6:659-664.

184. Kurosawa H, Tanaka H. Advances in immobilized cell culture: development of a co-immobilized mixed culture system of aerobic and anaerobic micro-organisms. Process Biochemistry Int. 1990; 25: 189-196

185. Kurosawa H, Nomura, N. Tanaka H. (1990) Ethanol production from starch by a co-immobilized mixed culture system of *Aspergillus awamori* and *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 1989; 33: 716-723

186. Martins dos Santos VAP, Vasilevska T, Kajuk B, Tramper J and RH Wijffels. Production and Characterization of Double-Layer Beads for Co-immobilization of Microbial Cells. Biotechnology Annual Review. 1997; 3:227-244

187. Field JA., Stams AJM, Kato M, Schraa G. Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. Antonie van Leeuwenhoek. 1995; 67:47-77

188. Zitomer DH, Speece RH.. Sequential environments for enhanced biotransformation of aqueous contaminants. Environm. Sci. Technol. 1993; 27:227-244

189. Wanner O, Gujer W. Competition in biofilms. Wat,. Sci Technol. 1984, 17:27-44

190. Wanner O, Gujer W., A multispecies biofilm model. Biotechnol. Bioeng. 1986; 28:314-328

191. Revsbech NP, Christensen PB, Nielsen LP. and Svrensen J. Denitrification in a trickling filter biofilm studied by a microsensor for oxygen and nitrous oxide. Water Research. 1989; 23:867-871

192. Kuenen JG, Jorgensen BB, Revsbech NP. Oxygen microprofiles of trickling filter biofilms. Water Research 1986; 20:1589-1598

193. Daalsgaard T, Revsbech N.P. Regulating factors of denitrification in Trickling filter biuofilms as measured with the oxygen /nitrous oxide microsensor. FEMS Microbiol. Ecol., 1992: 101:151-157

194. Watanabe S, Bang DY, Itoh K, Matsui K. Nitrogen removal from wastewater by a bioreactor with partially and fully submerged rotating biofilms. Wat. Sci. Technol. 1994; 29:431-438

195. Watanabe S, Masuda S. Nishidome K, Wantanwin C. Mathematical model of simultaneous carbon oxidation, nitrification and denitrification rotating biological contactors. Wat. Sci. Technol. 1984; 17:385-397

196. Gujer W, M Boller. A Mathematical model for rotating biological contactors. Proc. EWPCA Conference on Technical advances in biofilm reactors, Nice, France. 1989 : 69-89.

197. Hao X, Zhang Z. Experimental research of nitrogen removal in a model oxidation ditch and initial probe about its principle. China Environ. Sci., 1990; 10:440-445

198. Patrick WH. Nitrogen transformations in submerged soils, In: Nitrogen in Agricultural Soils, F.J. Stevenson (ed.), American Society of Agronomy Publisher, 1982, pp. 449-465, Madison, Wisconsin, USA

199. Blackburn TH, Blackburn ND, Jensen K, Petersen N.R. Simulation model of the coupling between nitrification and denitrification in a freshwater sediment. Appl. Environm. Microbiol. 1994; 60:3089-3095

200. Jensen K, Sloth N, Risgaard-Petersen N, Rysgaard S, Revsbech N.P. Estimation of nitrification and denitrification from microprofiles of oxygen and nitrate in model sediment systems. Appl. Environ. Microbiol. 1994; 60:2094-2100

201. Rijsgaard S, Risgaard-Petersen N, Nielsen LP, Revsbech N.P. Nitrification and denitrification in lake sediments measured by the N dilution technique and isotope pairing. Appl. Environm. Microbiol. 1993; 59:2093-2098.

202. Van Van de Graaf AA, Mulder A, de Bruijn P, Jetten MSM, Robertson LA and Kuenen JG. Anaerobic ammonium oxidation is a biologically-mediated process.

203. Van de Graaf AA, Mulder A, de Bruijn P, Jetten MSM, Robertson LA and Kuenen JG. Autotrophic growth of anaerobic ammonium oxidizing microorganisms in a fluidized bed reactor. Microbiology. 1996; 142:2187-2196

204. Martins dos Santos VAP, Tramper J, Jetten MSM; Kuenen JG, Wijffels RH. Simultaneous Autotrophic Nitrification and Anaerobic Ammonium Oxidation with Co-immobilized Micro-organisms. Appl. Environm. Microbiol., submitted

205. Brindle K, Stephenson T. The application of membrane bioreactors for the treatment of wastewaters. Biotechnol. Bioeng. 1996; 49:601-610

206. Livingston AG. Extractive membrane bioreactors: a new process technology for detoxifying chemical industry wastewaters. J. Chem. Technol. Biotechnol. 1994, 60:117-124

207. Smith CW, Di Gregorio D, Talcott RM. The use of ultrafiltration membranes for activated-sludge separation. In: Proceedigs of the 24th Annual Purdue Industrial Waste conference, pp. 1300-1310. 1969. Purdue University, West Lafayette, Indiana, USA

208. Van Dijk L., Roncken GCG. Membrane bioreactors for wastewater treatment. Proceedings of the 1996 Aquatech conference on Advanced Wastewater Treatment. September 23-25, Amsterdam, The Netherlands

209. Muller EB, Stouthammer AH, Varseveld HW, Eikelboom DH. Aerobic domestic wastewater treatment in a pilot plant with complete sludge retention by cross-flow filtration. Water Research. 1995; 29:1179-1189.

210. Chaize S, Huyard A. Membrane bioreactor on domestic wastewater treatment: sludge production and modeling approach. Membr. Sep. 1990; 23:1591-1600

211. Delanghe B, Nakamura F, Myoga H, Magara Y. Biological denitrification with ethanol in a membrane bioreactor. Environmental Technology 1994; 15:61-70

212. Barreiros AM, Crespo JPSG, Reis MAM. Biological denitrification in a membrane bioreactor. In: Proceedings of the 1996 Aquatech conference on Advanced Wastewater Treatment, September 23-25, Amsterdam, The Netherlands, pp. 401-403.

213. Chiemchaisri C, Wong YK, Urase T, Yamamoto K. Organic stabilization and nitrogen removal in a membrane separation bioreactor for domestic wastewater treatment. Filtrat. Sep., 1993; 30:247-252

214. Suwa Y, Suzuki T, Toyohara H, Yamagishi T, Urushigawa Y. Single-stage, single-sludge nitrogen removal by an activated sludge with cross-filtration. Water Research 1992; 26:1149-1157.

186. Martins dos Santos VAP, Vasilevska T, Kajuk B, Tramper J and RH Wijffels. Production and Characterization of Double-Layer Beads for Co-immobilization of Microbial Cells. Biotechnology Annual Review. 1997; 3:227-244

187. Field JA., Stams AJM, Kato M, Schraa G. Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. Antonie van Leeuwenhoek. 1995; 67:47-77

188. Zitomer DH, Speece RH.. Sequential environments for enhanced biotransformation of aqueous contaminants. Environm. Sci. Technol. 1993; 27:227-244

189. Wanner O, Gujer W. Competition in biofilms. Wat,. Sci Technol. 1984, 17:27-44

190. Wanner O, Gujer W., A multispecies biofilm model. Biotechnol. Bioeng. 1986; 28:314-328

191. Revsbech NP, Christensen PB, Nielsen LP. and Svrensen J. Denitrification in a trickling filter biofilm studied by a microsensor for oxygen and nitrous oxide. Water Research. 1989; 23:867-871

192. Kuenen JG, Jorgensen BB, Revsbech NP. Oxygen microprofiles of trickling filter biofilms. Water Research 1986; 20:1589-1598

193. Daalsgaard T, Revsbech N.P. Regulating factors of denitrification in Trickling filter biuofilms as measured with the oxygen /nitrous oxide microsensor. FEMS Microbiol. Ecol., 1992: 101:151-157

194. Watanabe S, Bang DY, Itoh K, Matsui K. Nitrogen removal from wastewater by a bioreactor with partially and fully submerged rotating biofilms. Wat. Sci. Technol. 1994; 29:431-438

195. Watanabe S, Masuda S. Nishidome K, Wantanwin C. Mathematical model of simultaneous carbon oxidation, nitrification and denitrification rotating biological contactors. Wat. Sci. Technol. 1984; 17:385-397

196. Gujer W, M Boller. A Mathematical model for rotating biological contactors. Proc. EWPCA Conference on Technical advances in biofilm reactors, Nice, France. 1989 : 69-89.

197. Hao X, Zhang Z. Experimental research of nitrogen removal in a model oxidation ditch and initial probe about its principle. China Environ. Sci., 1990; 10:440-445

198. Patrick WH. Nitrogen transformations in submerged soils, In: Nitrogen in Agricultural Soils, F.J. Stevenson (ed.), American Society of Agronomy Publisher, 1982, pp. 449-465, Madison, Wisconsin, USA

199. Blackburn TH, Blackburn ND, Jensen K, Petersen N.R. Simulation model of the coupling between nitrification and denitrification in a freshwater sediment. Appl. Environm. Microbiol. 1994; 60:3089-3095

200. Jensen K, Sloth N, Risgaard-Petersen N, Rysgaard S, Revsbech N.P. Estimation of nitrification and denitrification from microprofiles of oxygen and nitrate in model sediment systems. Appl. Environ. Microbiol. 1994; 60:2094-2100

201. Rijsgaard S, Risgaard-Petersen N, Nielsen LP, Revsbech N.P. Nitrification and denitrification in lake sediments measured by the N dilution technique and isotope pairing. Appl. Environm. Microbiol. 1993; 59:2093-2098.

202. Van Van de Graaf AA, Mulder A, de Bruijn P, Jetten MSM, Robertson LA and Kuenen JG. Anaerobic ammonium oxidation is a biologically-mediated process.

203. Van de Graaf AA, Mulder A, de Bruijn P, Jetten MSM, Robertson LA and Kuenen JG. Autotrophic growth of anaerobic ammonium oxidizing microorganisms in a fluidized bed reactor. Microbiology. 1996; 142:2187-2196

204. Martins dos Santos VAP, Tramper J, Jetten MSM; Kuenen JG, Wijffels RH. Simultaneous Autotrophic Nitrification and Anaerobic Ammonium Oxidation with Co-immobilized Micro-organisms. Appl. Environm. Microbiol., submitted

205. Brindle K, Stephenson T. The application of membrane bioreactors for the treatment of wastewaters. Biotechnol. Bioeng. 1996; 49:601-610

206. Livingston AG. Extractive membrane bioreactors: a new process technology for detoxifying chemical industry wastewaters. J. Chem. Technol. Biotechnol. 1994, 60:117-124

207. Smith CW, Di Gregorio D, Talcott RM. The use of ultrafiltration membranes for activated-sludge separation. In: Proceedigs of the 24th Annual Purdue Industrial Waste conference, pp. 1300-1310. 1969. Purdue University, West Lafayette, Indiana, USA

208. Van Dijk L., Roncken GCG. Membrane bioreactors for wastewater treatment. Proceedings of the 1996 Aquatech conference on Advanced Wastewater Treatment. September 23-25, Amsterdam, The Netherlands

209. Muller EB, Stouthammer AH, Varseveld HW, Eikelboom DH. Aerobic domestic wastewater treatment in a pilot plant with complete sludge retention by cross-flow filtration. Water Research. 1995; 29:1179-1189.

210. Chaize S, Huyard A. Membrane bioreactor on domestic wastewater treatment: sludge production and modeling approach. Membr. Sep. 1990; 23:1591-1600

211. Delanghe B, Nakamura F, Myoga H, Magara Y. Biological denitrification with ethanol in a membrane bioreactor. Environmental Technology 1994; 15:61-70

212. Barreiros AM, Crespo JPSG, Reis MAM. Biological denitrification in a membrane bioreactor. In: Proceedings of the 1996 Aquatech conference on Advanced Wastewater Treatment, September 23-25, Amsterdam, The Netherlands, pp. 401-403.

213. Chiemchaisri C, Wong YK, Urase T, Yamamoto K. Organic stabilization and nitrogen removal in a membrane separation bioreactor for domestic wastewater treatment. Filtrat. Sep., 1993; 30:247-252

214. Suwa Y, Suzuki T, Toyohara H, Yamagishi T, Urushigawa Y. Single-stage, single-sludge nitrogen removal by an activated sludge with cross-filtration. Water Research 1992; 26:1149-1157.

215. Wherle Werk Ag, referenz Thor Chemie GmbH, Speyer, Chemieabswasserreinigung, referenz Bayern Leder GmbH & Co. Neutraubling, lederabwasserreinigung, referenz Hausmuelldeponie Kahlenberg. Sickerwasserreinigung, 1995

216. Aya H. Modular membranes for self-contained reuse systems. Water Quality International 1994; 4:21-22

217. Knoblock MD, Sutton PM, Mishra PN, Gupta J, Janson A. Membrane biological system for the treatment of oily wastewaters. Wat. Environ. Res. 1994; 66:133-139

218. Timberlake DL, Strand S E, Williamson, K.J. Combined aerobic heterotrophic oxidation. Nitrification and denitrification in a permeable-support biofilm. Wat. Res., 1988, 12:1513-1517

219. Hirasa O, Ichijo H, Yamauchi A. Preparation of a new support for immobilization of activated sludge. J. Ferm. Bioeng. 1991; 71:376-378

220. Suzuki Y, Miyhara S, Tokeishi K. Oxygen supply method using a gas permeable film for watewater treatment. Wat. Sci. Technol., 1993; 28:244-250

221. Lemoine D, Jouenne T, Junter G-A. Biological denitrification of water in a two-chambered immobilized-cell bioreactor. Appl. Microbiol. Biotechnol. 1991; 36:257-264

222. Özoguz G, Räbinger N, Baumgärt. Membraneisatz zur Erhöhung der Nitrifikationsleistung durch getrennte Substratversorgung. Bioforum. 1994; 17:19-135

223. De Beer D, Schramm A, Santegoeds C, Kuhl M. A nitrite microsensor for profiling environmental biofilms. Appl. Environm. Microbiol. 1997; 63:973-977

224. Schramm A, Larsen LH, Revsbech NP, Ramsing. NB, Amann R, Schleifer KH. Structure and function of a nitrifying biofilm as determined by in-situ hybridization and the use of microelectrodes. Appl. Environm. Microbiol. 1996; 62:4641-4647

225. Uemoto H, Saiki H. Behaviour of immobilized *Nitrosomonas europaea* and *Paracoccus denitrificans* in tubular gel for nitrogen removal in wastewater. In: Immobilized cells: basics and applications (Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J, Eds). Elsevier Science, The Netherlands, 1996, pp. 696-702

226. Uemoto H, Saiki H. Nitrogen removal by tubular gel containing *Nitrosomonas europaea* and *Paracoccus denitrificans*. Appl. Environ. Microbiol. 1966, 62:4224-4228

227. Yamamoto K, Hiasa M, Mahmood T, Matsuo T. Direct solid-liquid separation using hollow fiber membrane in an activated sludge aeration tank. Wat. Sci. Technol. 1989; 21:43-54

228. Diels L, Van Roy S, Mergeay M, Doyen W., Taghavi S, Leysen R. Immobilization of bacteria in composite membranes and development of tubular membrane reactors for heavy-metal recuperation. In: Proceedings of the 3rd International Conference on Effective Membrane Processes. 1993, Volume 3, pp. 275-293

229. Jetten MSM, Horn S, van Loosdrecht MCM. Towards a more sustainable municipal wastewater treatment system. Wat. Sci. Technol. 1997, In press

230. Jetten MSM, Logemann S, Muyzer G, Robertson LA, de Vries S, van Loosdrecht MCM, Kuenen JG. Novel principles in the microbial conversion of nitrogen componds. Antonie van Leeuwenhoek 1997; 71:75-93.

231. Kuenen JG, Robertson LA. Combined nitrification-denitrification processes. FEMS Microbiol. Rev. 1994; 15:109-117

232. Robertson LA, Kuenen JG. Physiology of nitrifying and denitrifying bacteria. In: Microbial production and consumption of grennhouse gases: Methane, nitrogen oxides and halomethanes. Rogers, J., Whitman, W. (Eds.) Am. Soc. Microbiol. Press, Washington D.C., 1991,189-200

233. Cole JA., Biodegradation of inorganic nitrogen compounds. In: Biochemistry of Microbial Degradation, C. Ratledge (ed.), Kluwer Academic Publishers, Dordrecht, The Netherlands, 1994, pp.487-512

234. Kuenen JG, Robertson LA. Ecology of nitrification and denitrification. In: Symp. Soc. Gen. Microbiol. 1988, 42 de, Nitrogen and sulphur cycles, 161-218

235. Robertson LA, Kuenen JG. Combined heterotrophic nitrification and aerobic denitrification in *Thiosphera pantotropha* and other bacteria. Antonie van Leeuwenhoek, 1990; 57:139-152

236. Wherfritz JM, Reilly A, Spiro S, Richardson D.J. Purification of hydroxilamine oxidase from *Thiosphaera pantoropha*. FEBS Lett. 1993; 335:246-250

237. Van Niel EWJ, Arts PAM, Wesselink BJ, Robertson LA and Kuenen JG. Competition between heterotrophic and autotrophic nitrifiers for ammonia in chemostat cultures. FEMS Microbiology Ecology.1993;102: 109-118

238. Gupta SK, Raja SM and Gupta AB. Simultaneous nitrification-denitrification in a rotating biological contactor. Environ. Technol. 1994; 15:145 -153

239. Hippen A, Rosenwinkel K-H, Baumgarten G, Seyfried CF. Aerobic deammonification: a new experience in the treatment of wastewaters. In: Proceedings of the 1996 Aquatech conference on Advanced Wastewater Treatment, September 23-25, Amsterdam, The Netherlands, pp. 215-222.

240. Pel R, Oldenhuis R, Brand W, Vos A, Gotschal JC, Zwart KB. Stable isotope analysis of a combined nitrification-denitrification sustained by thermophilic methanotrophs under low-oxygen conditions. Appl. Environm. Microbiol. 1997; 63:474-481

241. Otte S, Grobben N, Robertson LA, Jetten MSM, Kuenen JG. Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions. Appl. Environm. Microbiol. 1996; 62:2421-2426

242. Van Niel EWJ, Braber KJ, Robertson LA and Kuenen JG. Heterotrophic nitrification and denitrification in *Alcaligenes faecalis* strain TUD. Antonie van Leeuwenhoek. 992; 62:231-237

243. Pot MA. Mogelijkheden tot optimalisatie van de biologische stikstofeliminarie. RWZI 200, rapport 94-09, 1995.

244. Davies K, Lloyd D, Boddy L. The effect of oxygen on denitrification in *Paracoccus denitrificans* and *Pseudomonas aeuroginosa*. J. Gen. Microbiol. 1989; 135:2445-51

245. Hochstein LI, Betlach M and Krtikos G. The effect on denitrification during steady-state growth of *Paracoccus halodenitrificans*. Archives Microbiology. 1984; 137:74-78.

246. Kawakami Y, Pacaud B, Nishimurea H. Inhibition of denitrification by oxygen in *Paracoccus denitrificans*. J. Ferm. Technol. 1985; 63:437-442

247. Watahiki M, Hyuichi H, Aida T. $N_2O$ accumulation and inhibition of $N_2O$ reduction by denitrifying *Pseudomonas sp.* 220A in the presence of oxygen. Agric.Biol. Chem. 1983; 47(9):1991-96

248. Tortoso AC, Hutchinson GL. Contributions of autotrophic and heterotrophic nitrifiers to soil NO and $N_2O$ emissions. Appl. Environm. Microbiol. 1990; 56:1799-1805

249. Arts PAM, Robertspon LA, Kuenen JG. Nitrificationand denitrification by *Thiosphaera pantotropha* chemostat cultures. FEMS Microbiol. Ecol. 1995; 18:305-315

250. Grobben N, Robertson LA, Kuenen JG. Nitrous oxide production by denitrifiers. Book of Abstracts 5th Netherlands Congress on Biotechnology. 1994, PJ-4.

251. Stouthammer AH. Dissimilatory reduction of oxidized nitrogen compounds: In Biology of anaerobic microorganisms. (Zhender, A.J.B. Ed.) 1988, John Wiley & Sons, New York, USA, pp 245-303

252. Anderson IC, and Levine JS. Relative rates of NO and $N_2O$ production by nitrifiers, denitrifiers and nitrate respirers. Appl. Env. Microbiol.. 1986; 51:938-945.

253. Carr GJ, Ferguson SJ. The nitric oxide reductase of *Paracoccus denitrificans*. Biochem. J. 1990; 269:423-429

254. Geywitz-Hetz SG, Schon G. $N_2O$ release of activated sludge under different conditions. In: Abstracts of the Beijrinck Centennial 1995, Delft University Press, Delft, The Netherlands, p. 205

255. Frunzke K, Zumft W. Inhibition of nitrous oxide respiration by nitric oxide in the denitrifying bacterium *Pseudomonas perfectomarina.* Biochim. Biophys. Acta. 1986; 852:119-125

256. Schulthess R, Wild D, Gujer W. Nitric and nitrous oxides from denitrifying sludges at low oxygen concentration. Wat. Sci. Technol. 1994; 30:123-132

257. Schulthess D, Gujer W. Release of nitrous oxide from denitrifying sludge. Verification and application of a mathematical model. Wat Res. 1996; 30:521-530

258. Ferguson SJ. Denitrification and its control. Antonie van Leeuwenhoek. 1994; 66:89-110

259. Payne WJ. Denitrification, Chinchester, John Wiley & Sons, 1981

260. Knowles R. Denitrification. Microb. Rev., 1982, 46:43-70

261. Alefounder PR, Greenfield AJ, McCarthy JE, Ferguson SJ. The basis for preferential electron flow to oxygen rather than nitrogen oxides in the denitrifying bacterium *Paracoccus denitrificans.* In: Microbial gas metabolism_ mechanistic, metabolic and biotechnologic aspecs. Poole, R.K., Dow, C.S. (eds.) London Academic Press. 1984; 225-230

262. Korner R, Benckiser G, Ottow J. Quantifizierung der Lachgas - Freisetzung aus Klaranlagen unterschiedlicher Verfahresnfuhrung. Abwasserreinigung. 1993; 40:514-525

263. Schon G, Bussmann M, Geywitz-Hetz G. Bildung von Lachgas mi belebten Schlamm aus Klaranlagen. Wasser Abwasser. 1994;135:293-301

264. Abeliovich A, Vonshak A. Anaerobic metabolism of *Nitrosomonas europaea*. Arch. Microbiol. 1992; 158:267-270

265. Bock E, Schmidt I, Stuven R, Zart D. Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. Arch. Microbiol. 1995; 163:16-20.

266. Freitag A, Rudert M, Bock E. Growth of *Nitrobacter sagilis* by dissimilatoric nitrate reduction. FEMS Microbial Lett. 1987; 48:105-109

267. Garrido JM, van Benthum WAJ, van Loosdrecht MCM, Heijnen JJ. Influence of dissolved oxygen concentration on nitrite accumulation in a Biofilm Airlift suspension reactor. Biotechnol. Bioeng. 1997; 53:168-178

268. Blackmer A, Bremner J, Scmidt E. Production of $N_2O$ by ammonium oxidizing chemoautotrophic microorganisms. Appl. Environm. Microbiol. 1980; 40:1060-1066

269. Poth M, Focht D. $^{15}N$ kinetic analyusis of $N_2O$ production by *Nitrosomonas europaea*: an examination of nitrifier denitrification. Appl. Environm. Microbiol. 1985: 49-1134-1141

270. Goreau T, Kaplan W, Wofsy S, McElroy M, Valoi F, Watson S. Production of $NO_2^-$ and $N_2O$ by nitrifying bacteria at reduced oxygen concentrations. Appl. Environm. Microbiol. 1980; 40:526-532

271. Boettcher B, Koops H.P. Growth of lithotrophic ammonia-oxidizing bacteria on hydroxilamine. FEMS Microbiol. Lett. 1994, 122:263-266.

272. Stuven R, Vollmer M, Bock E. The impact of organic matter on nitric oxide formation by *Nitrosomonas europaea*. Arch Microbiol. 1992; 158:439-443

273. De Bruijn P, Van der Graaf AA, Jetten MSM, Robertson LA, Kuenen JG. Growth of *Nitrosomonas europaea* in hydroxylamine. FEMS Microbiol. Lett. 1995; 125:179-184

274. Hooper AB. A nitrite reducing enzyme from *Nitrosomonas europaea*. Preliminary chracterization with hydroxylamine as electron donor. Biochim.Biophys. Acta. 1968; 162:49-65

275. Okabe S, Hiratria K, Ozawa Y, Watanabe Y. Spatial microbial distribution of nitrifiers and heterotrophs in mixed-population biofilms. Biotechnol. Bioeng., 1996, 50:24-35

276. Suthersand S, Ganczarczyk JJ. Inhibition of nitrite oxidation during nitrification. Some observations. Wat. Poll. Res. J. Canada. 1986; 21:257-266

277. Abeling U, Seyfried CF. Anaerobic-aerobic treatment of high strength ammonium wastewater- nitrogen removal via nitrite. Wat. Sci. Technol., 1992, 26:1007-10015

278. Turk O, Mavinic DS. Stability of nitrite build-up in an activated sludge system. J. Wat. Poll. Control. Fed. 1989; 61:1440-1448

279. Balmelle B, Nguyen KM, Capdeville B, Cornier JC, Deguin A. Study of the factors controlling the nitrite build-up in biological processes for water nitrification. Wat. Sci. Technol. 1992;26:1017-1025

280. Brouwer M. Stikstofverwijdering uit rejectie water met behulp van het SHARON proces. BSDL Thesis, Delft University of Technology. 1995, Delft, The Netherlands (as reported in Jetten et al., 1997)

281. Picioreanu C, Van Loosdrecht MCM, Heijnen JJ. Modeling of the effect of oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. Proceedings of the 3rd International IAQW special conference on biofilm systems, Copenhagen, 27-30 August 1996

282. Van de Graaf AA, Mulder A, Slijkhuis H, Robertson LA and Kuenen JG. Anaerobic ammonium oxidation In:Christiansen, C., Munck, L and Villadsen, J. (eds.. Proc. 5th European Congress on Biotechnology. Munckgaard International Publishers. Copenhagen, 1990; 1:388-391

283. Mulder A, van de Graaf AA, Robertson LA and Kuenen JG. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. 1995; 16:177-184

284. Broda E. Two kinds of lithotrophs missing in nature. Zeitschrift fur Allgemeine Mikrobiologie. 1977; 17:491-493

285. Strous M, van Gerven E, Kuenen JG, Jetten M. Effects of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (Anammox) Sludge. Appl. Environm. Microbiol., 1997; 63:2446-2448

286. Strous M, van Gerven E, Ping Z, Kuenen JG, Jetten MSM. Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (Anammox) process in different reactor configurations. Water Research, 1997; 31:1955-1963

287. Martins dos Santos VAP, Tramper J, Jetten MSM, Kuenen JG, Wijffels RH. Design and Scale up a Fully Autotrophic Nitrogen Removal process. Submitted.

288. Leenen EJ TM, Martins dos Santos VA.P, Grolle KCF., Tramper J, Wijffels RH. Characteristics of and selection criteria for support materials for cell immobilization in wastewater treatment. Wat. Res., 1996; 30: 2985-2996

289. Martins dos Santos VAP, Leenen EJTM., van Vliet T, Tramper J, Wijffels RH. 1997. Relevance of the Rheological Properties of Gel Beads for Their Mechanical Stability in Bioreactors. Biotechnol. Bioeng. 1997; 56:517-529

290. Gjaltema A, Vinke JL, van Loosdrecht MCM, Heijnen J.J. Abrasion of suspended biofilm pellets in airlift reactors: importance of shape, structure and particle concentrations. Biotechnol. Bioeng. 1997; 53:88-99

291. Gjaltema A, van Loosdrecht MCM., Heijnen JJ. Abrasion of suspended biofilm pellets in airlift reactors: effect of particle size. Biotechnol. Bioeng. 1997;55:206-21588-99

292. Gjaltema A, Van der Marek N, van LoosdrechtMCM, Heijnen JJ. Adhesion of and development of of biofilms on suspended carriers in air-lift reactors: influence of hydrodynamic conditions and surface characteristics. Biotechnol. Bioeng. 1997; 56: 345-356

293. Bryers JD, Banks MK. Assessment of biofilm ecodynamics: In: Physiology of Immobilized cells. de Bont, J., Visser, J., Matiasson, M., Tramper, J. (Eds.). 1990. Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 49-61.

294. Hüsken LE, Tramper J, Wijffels RH. Growth and eruption of gel-entrapped microcolonies. In: Immobilized cells: basics and applications (Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J, Eds). Elsevier Science, The Netherlands, 1996, pp. 336-340

295. Salmon, P.M. Mass transfer Phenomena in reactors containing enzymes or bacterial cells. PhD. thesis, 1989, Stanford University, USA

296. Vogelsang C, Østgaard K. Stability of alginate gels applied for cell entrapment in open systems. In: Immobilized cells: basics and applications (Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J, Eds). Elsevier Science, The Netherlands, 1996, pp. 213-220

297. Hunik JH, Tramper J. Large-scale productions of κ-carrageenan droplets for gel-bead production:Theoretical and practical limitations of size and production rate. Biotechnol. Progr. 1993;9:186-192

298. Wimpenny JWT, Colasanti R. A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. FEMS Microbiol. Ecol. 1997; 22:1-16

299. van Loosdrecht MCM, Eikelboom DE, Gjaltema A, Tijhuis L, Heijnen JJ. Biofilm structures. Wat. Sci. Technol. 1995;32:35-43

300. De Beer D, Stoodley P, Lewandowski Z. Effects of biofilm structures on oxygen distribution and mass transport. Biotechnol. Bioeng., 1994; 43:1131-1138

301. De Beer D, Stoodley P, Lewandowski Z. Liquid flow in heterogeneous biofilms. Biotechnol. Bioeng. 1994; 44:636-641

302. Stewart PS, Murga R, Srinivasan R, de Beer D. Biofilm structural heterogeneity visualized by three microscopic methods. Wat. Res. 1995, 29:2006-2009

303. Tijhuis L, Hijman B, Van Loosdrecht MCM, Heijnen JJ. Influence of detachment, substrate loading and reactor scale on the formation of biofilms in airlift reactors. Appl. Microbiol. Biotechnol. 1996; 45:7-17

304. Christensen FR, Holm KG, la Cour Jansen J. Biofilm structure - an important and neglected parameter in waste water treatment. Wat. Sci. Technol. 1989; 21:805-814

305. Zhang TC, Bishop PL. Structure, activity and composition of biofilms. Wat. Sci. Technol. 1994; 29:335-344

306. Leenen EJMT, Boogert AA, van Lammeren AAM, Tramper J, Wijffels RH. Dynamics o artificially immobilized *Nitrosomonas europaea*: effects of biomass decay. Biotechnol. Bioeng. (in press).

307. Martins dos Santos VAP, Tramper J, Wijffels RH. Towards An Integrated Approach For Oxidative And Reductive Biodegradation Processes. Case Study: Integrated Nitrogen Removal with Co-immobilized Microorganisms. Proceedings of the Symposium on Biodegradation of Organic Compounds, June 1996, Palma de Mallorca, Spain.

308. Bailey JE and Ollis D.F. Biochemical Engineering Fundamentals. 2nd ed. McGraw-Hill, New York, 1992.

309. Willaert RG., Reaction and diffusion in gel immobilized cell systems. Ph.D. thesis, Free University Brussels, 1996, Brussels, Belgium.

310. Tanaka H, Dunn IJ. Kinetics of biofilm nitrification. Biotechnol. Bioeng. 1982, 24:669-689

311. Rittmann B, Manem JA. Development and experimental evaluation of a steady-state, multispecies biofilm model. Biotechnol. Bioeng., 1992, 39:914-922.

312. Chen GH, Ozaki H, Terashima Y. Modelling of the simultaneous removal of organic substances and nitrogen in a biofilm. Wat. Sci. Technol. 1989; 21:791-804

313. Stewart PS. A model of biofilm detachment. Biotechnol. Bioeng., 1993, 41:111-117

314. Benefield L, Molz F. Mathematical simulation of a biofilm process. Biotechnol. Bioeng. 1985; 27:921-931

315. Annachatre AP, Khanna P. Unsteady-state biofilm kinetics. J. Environm. Eng. 1987, 113:429-423

316. Monbouquette HG, Sayles GD, Ollis DF. Immobilized cell biocatalyst activation and pseudo-state-behaviour: model and experiment. Biotechnol. Bioeng. 1990;35:609-629.

317. Martins dos Santos VAP, Verschuren P, van den Heuvel H, Tramper J, Wijffels RH. Substrate and product profiles across double-layer gel beads: modelling and experimental evaluation. Submitted.

318. Masuda S, Watanabe Y, Ishiguro M. Biofilm properties and simultaneous nitrification and denitrification in aerobic biological contactors. Wat. Sci. Technol. 1991; 23:1355-1363

319. Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J (Eds). Immobilized cells: basics and applications. Elsevier Science, The Netherlands, 1996, 818pp

This chapter has been published in: Applied Microbiology & Biotechnology, 1996, 45:447-453 by Martins dos Santos VAP, Bruijnse M, Tramper J, Wijffels RH.

## CHAPTER 3

# The Magic-Bead Concept: an Integrated Approach to Nitrogen Removal with Co-immobilized Microorganisms


## ABSTRACT

This paper describes both qualitative and quantitative aspects of simultaneous autotrophic nitrification and heterotrophic denitrification by, respectively, the nitrifier *Nitrosomonas europaea* and either of the denitrifiers *Pseudomonas denitrificans* or *Paracoccus denitrificans* co-immobilized in double-layered gel beads. The system is based on the establishment of well-defined oxic and anoxic zones within the cell supports and on physical separation of the nitrifying and denitrifying populations. Nitrification and denitrification rates were obtained from measured bulk concentrations and headspace analysis. The latter analyses showed that ammonia was primarily converted into molecular nitrogen. Nitrous oxide was not detected. High nitrogen removal rates (up to 5.1 mmol $N.m^{-3}_{gel}.s^{-1}$) were achieved in continuous reactors under aerobic conditions. The overall rate of nitrogen removal was controlled by the nitrifying step. The approach followed is, in principle, also suitable to the coupling of other oxidative and reductive bioprocesses having complementary metabolic routes. Two-stage bioconversion processes can be thus conducted as if single-staged, which results in more compact reactor systems.

## INTRODUCTION

Immobilized-cell processes have been receiving increasing attention in the last two decades. We have often used immobilized (de)nitrifying cells as a model system for the characterization of such processes (Wijffels *et al.* 1989,1990, 1991, 1995; Hunik *et al.* 1994). These studies have shown that high capacities can be reached in continuous (gas-lift) reactors containing immobilized nitrifying and denitrifying microorganisms, even under suboptimal environmental conditions such as low temperatures or concentrated streams. Complete nitrogen removal can be accomplished by coupling nitrification and denitrification. Integration of both processes into one single system is, in practice, difficult to achieve since different environments are required by the nitrifying (strict aerobic) and denitrifying (facultative anaerobic) cells. An attractive possibility would be the co-immobilization of these bacteria within gel beads in such a way that an outer nitrifying layer and a denitrifying core could be created, as suggested by Tramper (1984, 1987) and Martins dos Santos *et al.* (1992, 1993). In fact, in a nitrifying gel bead, the nitrifying microorganisms tend to concentrate in a thin biofilm just underneath the particle surface because of oxygen diffusion limitation, leaving the anoxic central part practically unused (Wijffels *et al.* 1991; Hunik *et al.* 1994). The establishment of oxic and anoxic zones within biocatalyst particles, biofilms or cell aggregates have been extensively described, both qualitatively (Kurosawa and Tanaka 1990) and quantitatively (Wijffels *et al.* 1991; Hooijmans *et al.* 1990a,b; de Beer *et al.*,1993). A general finding is that oxygen, owing to limitation of its uptake and diffusion, seldom penetrates more than a few hundred micrometres provided that it is the limiting substrate. Hence, if suitable denitrifying bacteria are present and organic substrates available, denitrification may occur within the core of these beads. The product of the first microorganism is the substrate for the second one, and this two-stage bioconversion system could thus be conducted as if single-staged. However, to construct such a system, the two species must be physically separated as they compete for oxygen uptake under aerobic conditions. If both microorganisms are simply mixed and directly immobilized in a gel bead, the fast-growing denitrifying cells would overgrow the slow-growing nitrifying ones in the presence of an organic carbon source. Kurosawa and Tanaka (1990) have addressed this problem for mixed cultures of obligatory aerobes and facultative anaerobes.

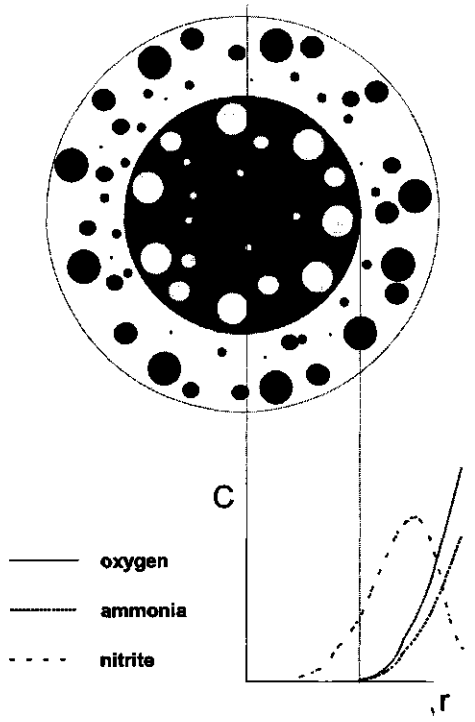


Figure 1 - Schematic representation of the "magic bead concept" le concentration, r radius). In the outer layer nitrification occurs. In the core denitrification takes place. Outer region: $NH_4^+$ + 3/2 $0_2$ → $2N0_2 + 2H^+ + H_2O$; inner core: $8NO_2 + 3CH_3COOH$→ $4N_2 + 6CO_2 + 2H_20 + 80H^-$

Physical separation of the two microbial populations (forced "habitat segregation") thus gives an important advantage to the nitrifiers and avoids the possibility that these cells are outcompeted by the denitrifying cells. This can be achieved by immobilization of the pertinent bacteria in double-layer beads, as shown in Fig. 1. Nitrification is accomplished in the outer layer and denitrification takes place in the bead core. Besides the obvious advantage of integrating two steps into one, two redundant steps can be avoided with substantial economy of oxygen and carbon. In a two-stage process, nitrite is normally first converted into nitrate at the expense of oxygen. The nitrate is, in the following stage (in the first step), reconverted into nitrite with carbon as co-substrate. These two steps can thus be circumvented by integration of both processes. In addition, the acid produced in the nitrifying step is partially compensated by the base resulting from denitrification.

This paper describes both qualitative and quantitative aspects of simultaneous nitrification and denitrification with the nitrifier *Nitrosomonas europaea* and either one of the denitrifiers *Pseudomonas* spp. or *Paracoccus denitrificans* co-immobilized in

double-layer gel beads under aerobic conditions in a continuous air-lift reactor. This system is used as a model for the study of integrated nitrogen removal with co-immobilized microorganisms. We will be referring to this as the magic bead concept.

## MATERIALS AND METHODS

### Organisms and culture conditions

*Nitrosomonas europaea* (ATCC 19718) was cultivated and collected for immobilization as earlier described (Hunik *et al.* 1994). *Pseudomonas* spp. LMD 84.60 (formerly designated *Pseudomonas denitrificans)* and *Paraccocus denitrificans* (LMD 22.21) were obtained from the Delft Culture Collection (The Netherlands). The cells were stored as glycerol stocks (ratio cell suspension/glycerol 1: 1) in 1-ml cryotubes (Nunc) at – 80°C. The denitrifying biomass used in the experiments was produced in a batch culture. The culture medium contained (in g/l) bactopeptone 6.0, beef extract 2.13, yeast extract 3.0 and glucose 1.25. For immobilization, three 450-ml samples of culture medium in three 2-1 conical flasks were each inoculated with 1 ml glycerol stocks of denitrifying cells. Glycerol stocks were thawed on ice. The cultures were grown at 30°C for 3 days in an incubator with shaking.

### Immobilization procedure

Cell suspensions of nitrifying and denitrifying bacteria were (separately) centrifuged for 10 min at 16 800 *g* in a GSA/Hb-4 rotor of a Sorvall Rc-5b centrifuge (Wilmington, Del., USA) at 5'C. The cell pellets were washed by resuspension in a 0.9% (w/w) NaCl solution and centrifuged again. For experiments with immobilized denitrifying microorganisms alone, the denitrifying-cell suspension was added to a dextran solution (Sigma, St Louis, USA, molecular mass 5 40 MDa) containing KCl so that a final solution of 10% (w/w) dextran and 1 M KCl was obtained. The resulting mixture was extruded dropwise through a hollow needle into a gently agitated 0.75% (w/w) κ-carrageenan solution (Genugel X0828, A/S Kobenhavns Pektinfabrik, Lille Skensved, Denmark). The beads obtained consisted of a liquid dextran core surrounded by a solid carrageenan envelope. Core and total bead diameter was 1.84 $\pm$ 0.15 mm and 3.90 $\pm$ 0.11 mm, respectively. These liquid-core beads were relatively weak and easily burst when they were operated in a continuous reactor (see section Results and Discussion). Therefore, for experiments with co-immobilized nitrifying and denitrifying cells, solid alginate was used as the inner support material instead. For this, the denitrifying suspension was added to an alginate (Protanal LF 10/60, Protan, Norway) and KCl solution so that a final mixture of 3% (w/w) alginate and I M KCl was obtained. The *Ns. europaea* cell suspension was added to a κ-carrageenan solution containing $CaCl_2$

and $BaCl_2$ so that the final concentrations were 1.5% (w/w), 0.08 M and 0.02 M respectively for carrageenan, $CaCl_2$ and $BaCl_2$. The immobilization procedure was further accomplished as described above. The final beads with co-immobilized *Ns. europaea* and *Pseudomonas* spp. had a total diameter f 3.8 $\pm$ 0.1 mm and a core diameter of 1.9 + 0.2 mm. The beads containing *Ns. europaea* and *Pa. denitrificans* had an outer diameter of 4.03 $\pm$ 0.23 mm and a core diameter of 2.12 $\pm$ 0.09 mm. The whole immobilization procedure (except centrifugation) was executed in a laminar-flow cabinet to provide aseptic conditions. All the objects and solutions used were sterilized at 121°C for an adequate time. The gels were sterilized at 115°C for a maximum of 15 min to avoid damage of the polymer structure and, as a consequence, excessive decrease of viscosity and gelation properties.

**Immobilized-cell cultivation**

***Denitrifying bacteria***

To check the capacity of the denitrifying cells to carry out denitrification when immobilized, *Pseudomonas* spp. and *Pa. denitrificans* cells were (separately) entrapped in the core of double-layer beads (as described above) and these (about 10 ml) were placed in a 50-ml flask, operated as a continuous reactor. Gentle agitation was provided by a shaking incubator at 30°C and the pH was maintained at 7.7 by a pH controller (LH Fermentation 500, series 111) using 0.25M HCl. The content of the reactor was continuously sparged with nitrogen to provide anoxic conditions. The medium (continuously supplied at a dilution rate of 1.1 x $10^{-5}$ $s^{-1}$) contained (I ') $K_2HP0_4$ (4.6 mmol), $KH_2PO_4$ (2.2 mmol), KCl (10 mmol), $CH_3COOH$ (20mmol), $NH_4Cl$ (0.5mmol), MgSO4-7H 20 (1.6 mmol), $KNO_2$ (3.0 mmol) and 2 ml Vishniac and Santer trace elements solution (Vishniac and Santer 1957). The medium was filter-sterilized using a Sartobran pH capsule, pore size 0.45 x 0.2 mm (Sartorius AG, Göttingen Germany). At the end of the run, the beads were collected, washed twice with 0.9% KCl (w/w) for about 15 min to remove the medium still contained in the beads and finally transferred (aseptically) to a 300-ml closed serum flask. A batch experiment was then executed as described in the subsection *Batch experiments* below, with a bead load of 10% (v/v).

*Co-immobilized cells*

To check whether co-immobilized *Ns. europaea and Pseudomonas* spp. were able to carry out simultaneous nitrification and denitrification, a batch experiment was carried out with freshly made double-layered beads as described in the subsection *Batch experiments.*

After the batch experiment, a continuous set-up was prepared. A bubble-column reactor

(150 ml working volume) was loaded with new double-layer gel beads (25% v/v) containing co-immobilized *Nitrosomonas europaea* and *Pseudomonas* spp. The reactor was supplied with sterilized medium, at a dilution rate of $5.56 * 10^{-4}$ $s^{-1}$ and contained the following (per l): $K_2HPO_4$ (4.6 mmol); $KH_2PO_4$ (2.2); KCl (10 mmol); $CH_3COOH$ (10 - 20 mmol); $(NH_4)_2SO_4$ (5 - 10 mmol); $MgSO_4.7H_2O$ (1.6 mmol) and 2 ml trace-elements solution (Vishniac & Santer, 1957). Temperature was kept at 30°C and the pH controlled (LH Fermentation 500 Series III) to 7.5 with KOH and HCl (both 0.25 M). Influent and effluent samples were taken daily for analysis of acetate and nitrogenous compounds. Air was supplied at a superficial velocity of about $3.5 * 10^{-2}$ $m.s^{-1}$, sufficient to maintain the dissolved oxygen concentration above 80% air saturation. The reactor was run for 12 days. To check if ammonia was completely converted to molecular nitrogen, the feed was stopped at the end of the run and the accumulation of molecular nitrogen and the consumption of oxygen in the reactor head space were followed in time.

In this and other preliminary runs with a bubble column frequent pH oscillations were observed (see section Results and Discussion). Therefore, continuous cultivation of co-immobilized *Nitrosomonas europaea* and *Paracoccus denitrificans* was executed in an internal air-lift reactor instead. The reactor (465 ml working volume and 256 ml head space) was loaded with 70 ml (15% v/v) double-layer carrageenan/alginate beads. Initial biomass concentrations were estimated at $1.05 * 10^{-2}$ $kg.m^{-3}$ and $2.2 * 10^{-1}$ $kg.m^{-3}$ for *Ns. europaea* and *Pa. denitrificans*, respectively. These estimates were obtained from oxygen consumption rates as described elsewhere (Martins dos Santos *et al*, 1996). Medium was supplied at a dilution rate of $1.4 * 10^{-4}$ $s^{-1}$ and contained, in addition to the medium above, $CaCl_2$ (0.2 $mmol.m^{-3}$) to maintain the mechanical integrity of the alginate core of the double-layer beads. Tris buffer (5 mM) substituted potassium buffer to avoid possible precipitation of calcium-phosphate salts. KCl was added to a concentration of 15 mM. Ammonium and acetate concentrations were both set at 5 $mmol.l^{-1}$. Temperature and pH were regulated in the same way as above. Air was supplied at a superficial velocity of $2.2 * 10^{-2}$ $m.s^{-1}$ which resulted in a gas hold-up of about 3.5% (v/v). The reactor was run for 30 days during which both influent and effluent were sampled daily. The gas phase of the reactor head space was analyzed daily as well. For headspace analysis, the reactors were switched into batch mode after flushing first for 45 min with a mixture of helium (79%) and oxygen (21%) to remove residual nitrogen. During the batch operation the gas mixture was continuously recirculated through the reactor at the same rate as in the continuous mode. Immediately

after switching to batch, 1 ml of methane was injected into the system as reference. The gas phase was analyzed every 3 - 4 minutes during the initial phase of the batch and each 15 minutes during the rest of the batch operation (two to three hours in total). Results were corrected for the volume withdrawn by sampling. At the beginning and at the end of the intermittent batch period, liquid samples were taken for analysis of ammonium, acetate and nitrite.

***Batch experiments***

The experiments were carried out in 250 ml serum flasks tightly closed with thick butyl-rubber septa. Hundred ml of medium (above described both for denitrifying cells alone and for co-immobilized cells) were added to the flasks. The media contained 10 mM nitrite and 10 mM acetate for experiments with denitrifying cells, and 10 mM ammonia and 10 mM acetate when co-immobilized *Nitrosomonas europaea* and *Pseudomonas* spp. cells were used. After sterilization of the flasks, double-layer beads containing denitrifying or both nitrifying and denitrifying cells were added. In the batch experiment done with freshly prepared beads with co-immobilized nitrifying and denitrifying bacteria, 64 ml beads (39% v/v) were added to the flask while for the batch experiments done after the continuous runs about 11 ml beads (10% v/v) were added. The flasks were placed in a shaking incubator at 30°C in a horizontal position to increase the gas/liquid contact area and flushed with helium for about 15 minutes, during which the nitrogen and oxygen concentrations were measured until they were considered to be low enough (< 1%). In the experiments with denitrifying cells 10 ml methane was injected at time zero. Methane was used as reference in the gas-phase measurements. For co-immobilized cells, together with methane, oxygen was injected in such a way that 20% (v/v) oxygen concentration was achieved. Whenever necessary (that is, if the oxygen tension came under 3-4 % (v/v)), oxygen was injected during the experiment. Gas samples were taken every five minutes and immediately analyzed. After every sampling, helium was injected in the reactor in the same amount of the sampled gas to correct for pressure differences. Results were furthermore corrected for the overpressure that resulted from injection of oxygen and methane and for the dilution that resulted from sampling. Accumulation of nitrogenous gas products and consumption of oxygen were followed in time. Liquid samples were also taken at the beginning and the end of the run for analysis of ammonia, nitrite, nitrate (as control) and acetate.

**Analytical methods**

Ammonia, nitrite and nitrate concentrations in batch media, and in the influent and effluent of the continuous reactors were measured by spectophotometry using a

continuous-flow analysis (Autoanalyser SB40, Skalar Methods) in the same way as described before (Hunik *et al*, 1994). Influent and effluent acetate concentrations were measured by gas chromatography for which a 2 m (2 mm inner diameter) glass column (10% Fluorad FC 431 on a Supelco-port 100-200 mesh) was used. The carrier gas was nitrogen at 30 ml/min, saturated with formic acid. The temperatures of the injection port, oven and detector were 200, 130 and 280°C, respectively. Samples were diluted with formic acid until the acetate concentration was less than 2 mg/l and the pH less than 2. The headspace in the batch experiment was analyzed by gas chromatography (Chrompack GC 9001 equipped with a thermal conductivity detector). Gas samples were taken with a pressure lock SGE 300 ml syringe with a 23G needle and a (SGE, series II) repeating adapter. Oxygen, nitrogen and methane were measured using a Molsieve column (80-100 mesh, 2m length and 3.18 mm inner diameter). Nitrous oxide and carbon dioxide were measured using a Hayesept Q column (80-100 mesh, 1m length and 3.18 mm inner diameter). Temperatures of the injector port, oven and detector were 100, 70 and 130 °C, respectively. Carrier gas: He at 20 ml/min.

## RESULTS

### *Continuous denitrification*

To check their ability to strains were immobilized denitrify, both denitrifying (separately) in the core of double-layer gel beads and cultivated anaerobically for 11 days under wash-out conditions.

After 8 days of cultivation, the nitrite-reduction rate for *Pseudomonas* spp. stabilized at 15 mmol $^{-1}.m^{-3}_{gel}$ (20% gel load, see Fig. 2). At this stage, the nitrite conversion was close to 100% as only traces of nitrite were found in the effluent and practically no acetate was left. For *Pa. denitrificans* these rates were well below the ones for *Pseudomonas* (Fig. 2).

After the first 24 h of the run, gas bubbles could be observed in the liquid dextran core of the beads, both for *Pa. denitrificans* and *Pseudomonas* spp. During the experiments the bubbles expanded and eventually this resulted in bursting of some of the beads, especially in the experiment with *Pseudomonas* spp. At the end of the continuous run with *Pa. denitrificans* a short batch cultivation with the collected beads was carried out for gas- and liquid-phase analysis. This was not possible to do with the beads from the immobilized *Pseudomonas* sp. since most of them had burst by that time. The net nitrogen gas accumulation (Fig. 3) and nitrite disappearance were followed over time. The accumulation of molecular nitrogen approached the total nitrite consumption. There was no $N_20$ detected in the gas phase.

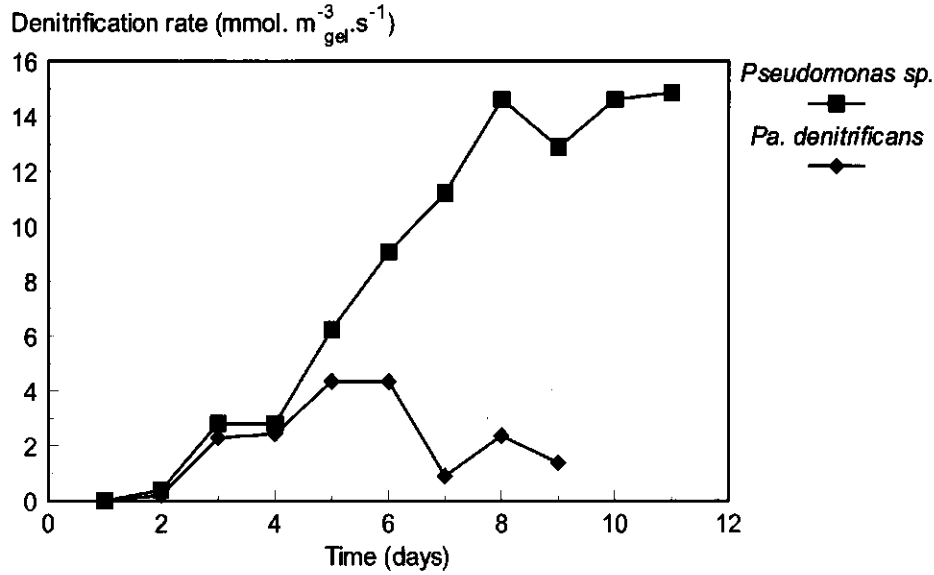


Figure 2 – Continuous denitrification with immobilized *Pseudomonas* spp. and *Paracoccus denitrificans* cells in a bubble column; $D = 1.11 * 10^{-3}\ s^{-1}$, gel load 20%.

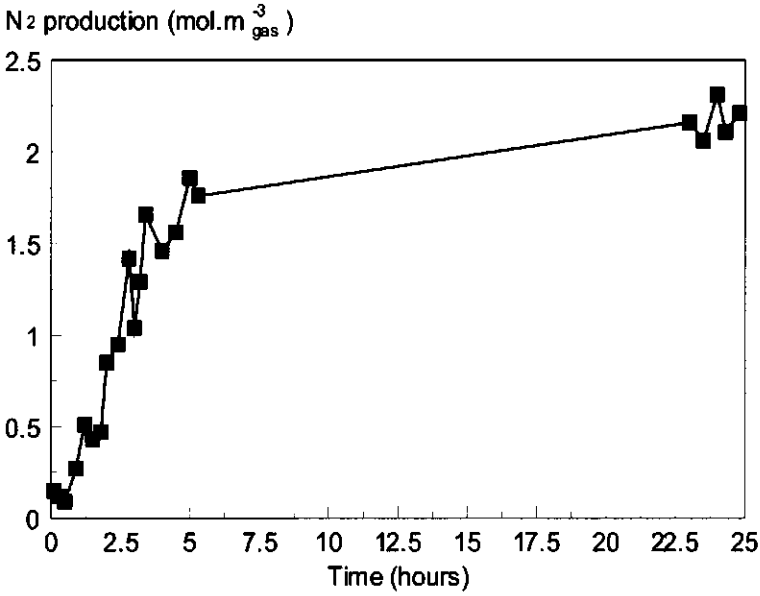


Figure 3 - Batch experiment with *Pseudomonas* spp. immobilized in the core of double-layer beads (gel load 10% v/v).

***Simultaneous nitrification and denitrification***

The next step was to check whether co-immobilized nitrifying and denitrifiying (both strains) microorganisms were able to nitrify and denitrify simultaneously. A batch experiment with *Ns. europaea* and *Pseudomonas* spp. freshly co-immobilized in alginate/ carrageenan beads was carried out and the gas phase was analysed to test whether ammonia was indeed converted into molecular nitrogen (Fig. 4). Several oxygen injections (indicated by the arrows) were necessary to provided enough oxygen for the process. The nitrogen production increased substantially especially after the second injection of oxygen. Unlike the process of denitrification alone, no gas bubbles were detected in the bead cores.

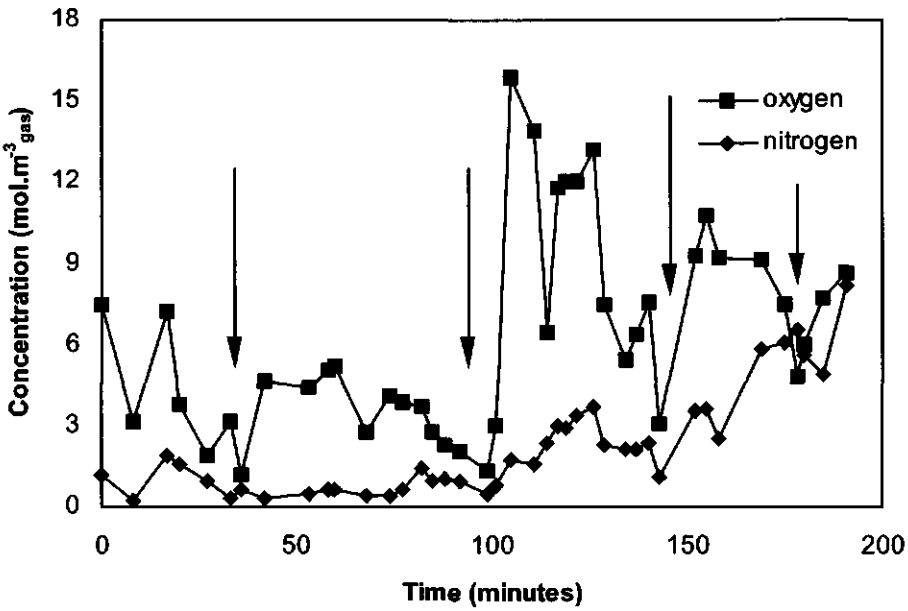


Figure 4 - Batch experiment with co-immobilized *Nitrosomonas europaea* and *Pseudomonas* spp. (gel load 39% v/v). Arrows oxygen injections

Subsequently new double-layer beads with co-immobilized *Ns. europaea* and *Pseudomonas* spp. were continuously cultivated in a bubble-column reactor for 12 days under aerobic conditions. The initial influent ammonia concentration was 5 mM and this was adjusted during the run so as to keep the effluent ammonia concentration always above 2 mM to prevent ammonia limitation. Nitrification and denitrification rates (Figure 5) were calculated on the basis of daily measured influent and effluent concentrations of ammonia and nitrite.

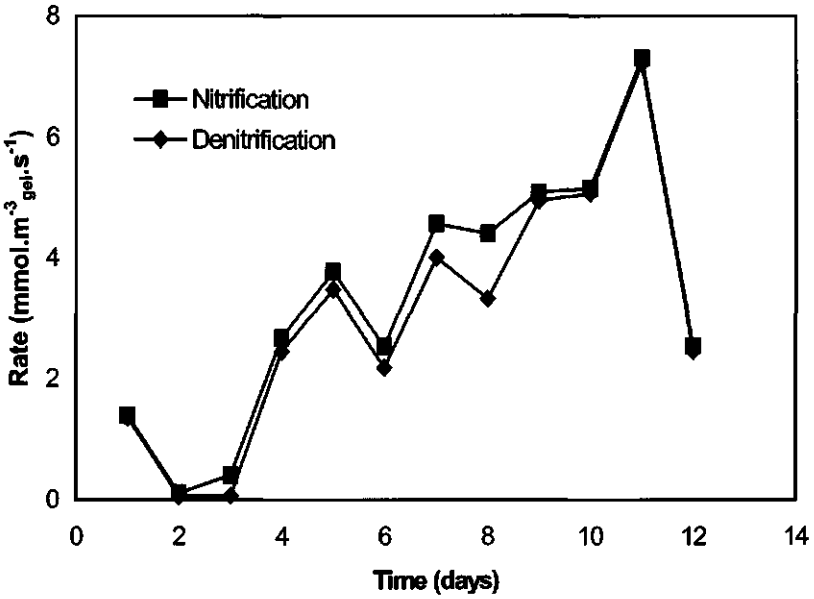


Figure 5 - Continuous nitrification and denitrification with co-immobilized *Ns. europaea* and *Pseudomonas* spp. in a bubble column; D=5.56 x $10^{-4}$ $s^{-1}$, gel load 25 %.

Only residual amounts of nitrite were found in the reactor outlet (less than 0.2 mM). The effluent acetate concentration was negligible after 4 days. The nitrification and denitrification rates determined were always very close to each other and, towards the end of the experiment (days 10-11), nitrogen removal capacities of about 5.1 mmol $m^{-3}_{gel}.s^{-1}$ (gel load 25%) were achieved. No gas bubble formation was observed. On days 2 and 11 sudden pH oscillations (down to pH values of 5.5-6.0) caused a sharp decrease in removal rates (Fig. 5). Smaller but frequent fluctuations in the pH were often observed during the run as well. It thus appeared that the mixing properties in the reactor were not good enough for adequate pH control.

To check whether ammonia was completely converted to molecular nitrogen, the beads of the continuous reactor were collected at the end of the run (day 12) and a batch experiment was done with headspace analysis. Figure 6 shows that molecular nitrogen accumulated steeply in the reactor headspace after injection of ammonium and acetate (C/N = 2 mol/mol) for the first 7 h, after which it stabilized at about 1.1 mmol $dm^{-3}$ gas. Ammonia was totally consumed at this point as the total nitrogen produced matched the amount of ammonia converted, roughly corrected for estimated fixation into biomass. No nitrous oxide was formed.

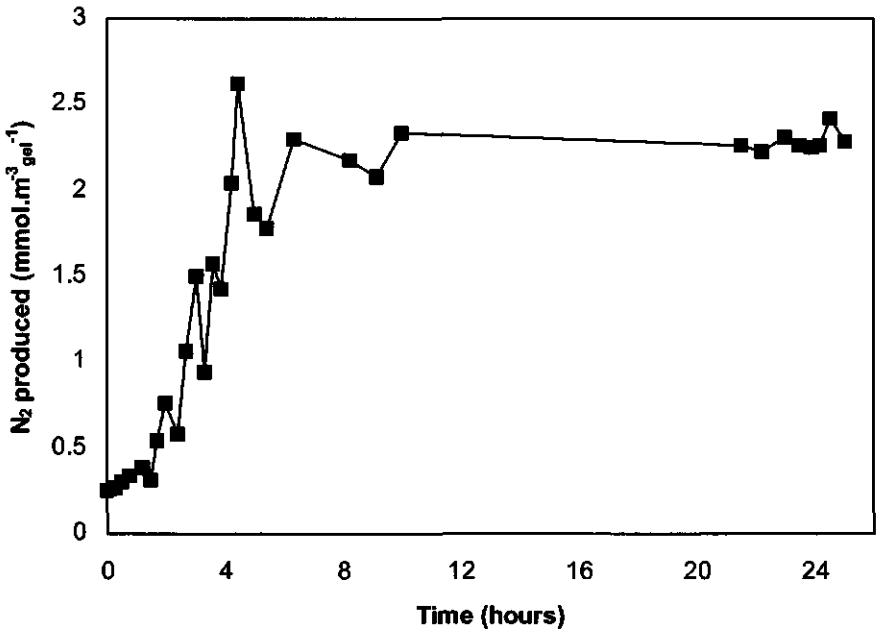


Figure 6 - Batch nitrification and denitrification with co-immobilized cells (gel load 10%) at the end of the continuous run shown in Figure 5.

With the information obtained from this set of experiments, an improved reactor system (air-lift) was used to study integrated nitrification and denitrification with co-immobilized *Ns. europaea and Pa. denitrificans.* Nitrification rates were calculated on the basis of influent and effluent nitrogenous concentrations whilst denitrification rates were determined by the gas production rates measured in the reactor headspace. In Fig. 7 the results of this experiment are presented. Nitrification and denitrification rates were again always close to each other during this 30-day run. An apparently steady state was attained about 10 days after start-up at a level of approximately 1.2 mmol. $m^{-3}_{gel}.s^{-1}$. Similarly to the experiment with *Nitrosomonas* and *Pseudomonas,* practically no nitrite was found in the effluent and no nitrous oxide production was detected during the run.

***Nitrogen balances***

Nitrogen fixed into biomass was estimated as being about 1.6% and 6% of the total ammonia converted for *Ns. europaea,* and for both *Pseudomonas* spp. *and Pa. denitrificans* respectively.

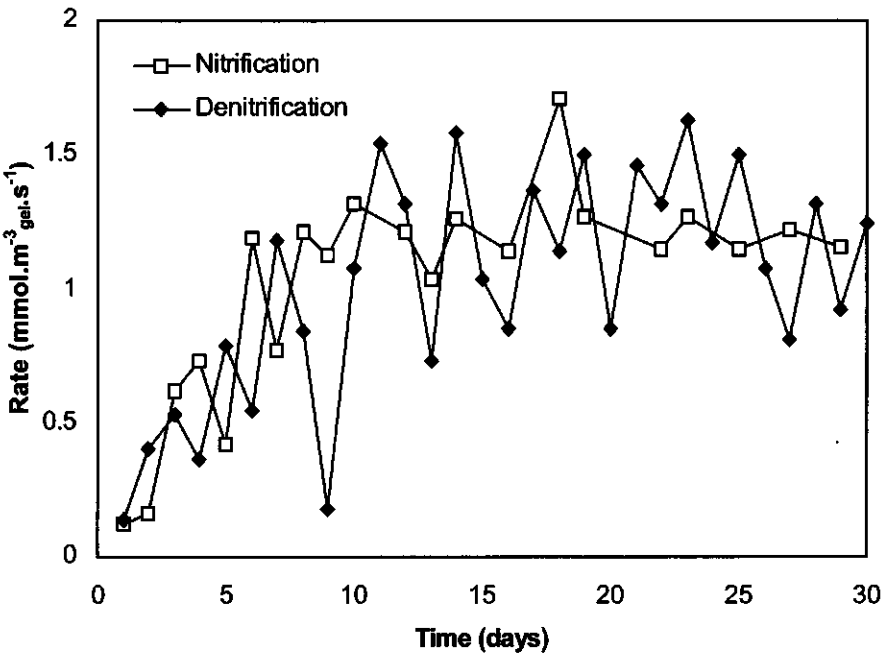


Figure 7 - Continuous nitrification and denitrification with co-immobilized *Ns. europaea* and *Pseudomonas* spp. in an air-lift reactor; D=1.4 x $10^{-4}$ $s^{-1}$,gel load 15%.

These figures were calculated from the corresponding yields on N of these strains. These were 2.16 g biomass mol $N^{-1}$ (Hunik *et al.* 1994), 7.39 g biomass mol N (Robertson *et al.* 1989) and 3.07 g biomass mol $N^{-1}$ (van Varseveld 1979) for *Ns. europaea, Pseudomonas* spp. and *Pa. denitrificans* respectively. The yield of *Ns. europaea* includes an increase of about 30% in relation to the yield reported by Hunik *et al.* (1994) to account for the presence of acetate, as reported by van Niel (1991). The formula $CH_20_{0.5}N_{0.25}$ was used for the three strains as an average biomass "composition" often quoted in the literature (Schlegel 1986). The nitrogen entering and leaving the reactor was balanced both for co-immobilized *Ns. europaea* and *Pseudomonas* spp. and for *Ns. europaea* co-immobilized with *Pa. denitrificans.* In both experiments the nitrogen losses were negligible.

## DISCUSSION

The aim of this work was to investigate the possibilities of integrating nitrification and denitritication in a compact, single-reactor system using microorganisms co-immobilized in double-layer beads. For this, we checked first whether denitrifying microorganisms were able to denitrify when immobilized in the core of double-layer beads. The results obtained show that heterotrophic denitrification did indeed occur in

the core of the double-layer gel beads both with *Pa. denitrificans* and *Pseudomonas* spp. Furthermore, these results indicate that nitrite, under the experimental conditions used, was completely reduced to molecular nitrogen. The denitrification rates attained are comparable to those described in the literature (Nilsson *et al.* 1980; Nilsson and Ohlson 1982; Wijffels *et al.* 1990; Kokufuta *et al.* 1987).

The next step was to investigate whether the co-immobilized nitrifying and denitrifying cells were able to nitrify and denitrify simultaneously. In all the experiments with co-immobilized microorganisms ammonium was converted to molecular nitrogen. The intermediary product, nitrite, was not detected in any of the experiments, so it is reasonable to conclude that the nitrite formed from the oxidation of ammonia by the *Ns. europ ea* cells was immediately decomposed via dissimilatory reduction by the denitrifying cells (either *Pa. denitrificans or Pseudomonas* spp.). The maximum nitrogen removal rate attained was about one-third of that obtained in the denitrifying experiments described above. This suggests that, in the integrated process, the nitrification rate limit the overall nitrogen conversion since the denitrifiers are dependent on the nitrite produced by nitrifying bacteria. This is confirmed by the fact that gas bubbles were not detected during the experiment, in contrast to the run with *Pseudomonas* spp. alone. Continuous cultivation of co-immobilized cells under the same conditions as above but using a medium lacking acetate resulted in accumulation of nitrite at approximately the same rate as the rate of ammonia consumption (results not shown). This therefore indicates that the nitrifying cells were not responsible for the degradation of nitrite. Heterotrophic nitrification was very likely unimportant in this system because these rates tend to be two or three orders of magnitude lower than those of autotrophic nitrification (Robertson *et al.* 1989; Robertson and Kuenen 1990). Therefore, since no nitrite or nitrate was added to the media, these results clearly indicate that coupled autotrophic nitrification and heterotrophic denitrification took place in the reactor under aerobic conditions.

Very likely this can be attributed to the establishment of aerobic and anaerobic regions within the beads and segregation of the nitrifying and denitrifying populations. By exploiting these characteristics, a single, compact system for integrated nitrogen removal at high rates can be developed. The ammonia conversion rates obtained were higher than those reported for nitrification alone with immobilized cells (Wijffels *et al.* 1989; Hunik *et al.* 1994; van Ginkel *et al.* 1983) or ammonia removal in conventional activated-sludge processes (EPA 1975), although somewhat lower than those for fluidized-bed and fixed-bed systems with attached biofilms (Tijhuis 1994; Gupta *et al.* 1994).

We are, at present, studying this system in more detail and deriving a mathematical model that enables prediction of transient phenomena and steady states at both bead and reactor level. Quantitative determination of the biomass distribution and substrate profiles within the beads is required for validation of the model and for further characterization of the system. Besides its environmental importance, characterization of a well-defined system provides fundamental insight into the use of co-culture systems for integration of oxidative and reductive biological processes. In fact, provided that any incompatibilities are not too strong (different pH, temperature, nutrients, substrate or byproduct toxicity) between two (or more) co-immobilized cell strains or consortia, this concept could be applied to the coupling of oxidative and reductive processes within a single, compact reactor system. Possible applications could be, for instance, biodegradation of xenobiotics and recalcitrant compounds such as dichlorodiphenyltrichloroethane (Beunink and Rehm 1990), 4-chloro-2-nitrophenol (Beunink and Rehm 1988) 2,4,6-trichlorophenol (Gardin and Pauss 1994) and 2,3,6-trichlorobenzoic acid (Gerritse and Gottschal 1992) and other aromatic pollutants (Field *et al.* t995). In all these studies, biodegradation is reported to occur at least in two sequential steps where both aerobic and anoxic conditions are required.

## ACKNOWLEDGEMENTS

We are grateful to Dr. Lesley Robertson of the Delft University of Technology, Kluyver Laboratory for Biotechnology, for kindly providing the denitrifying cells and for very valuable discussions. Thanks are due to Wim Roelofsen from the Microbiology Department of the Wageningen Agricultural University for help with the chromatographic analysis. We also acknowledge the financial support given by the Junta Nacional de Investigação Cientifica e Tecnológica (JNICT), Portugal (BD/1505/91 IF).

## REFERENCES

Beer D de, Van den Heuvel JC, Ottengraaf SPP (1993) Microelectrode measurements of the activity distribution in nitrifying bacterial aggregates. Appl Environ Microbiol 59:573-579

Beunik H, Rehm HJ (1988) Synchronous anaerobic and aerobic degradation of DDT by an immobilized mixed culture system. Appl Microbiol Biotechnol 29:72-80

Beunik H, Rehm HJ (1990) Coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol by a co-immobilized mixed culture system. Appl Microbiol Biotechnol 34:108-115

EPA (US Environmental Protection Agency) (1975) Process design manual for nitrogen control. USEPA, Washington D.C., USA.

Field JA, Stams AJ, Kato M, Schraa G (1995) Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. Antonie van Leeuwenhoek 67:47-77

Gardin H, Pauss A (1994) Co-immobilization of aerobic and anaerobic bacterial communities in a mixed K-carrageenan/gelatin gel for the degradation of 2,4,6-trichlorophenol. In: Lacroix C, Vuillemard J-C (eds) Proceedings of International Workshop on Bioencapsulation. 1V. Quebec, Canada. pp 93-97

Gerritse J, Gottschal JC (1992) Mineralization of the herbicide 2,3,6-trichlorobenzoic acid by a coculture of aerobic and anaerobic bacteria. FEMS Microbiol Ecol 10:89-98

Ginkel CG van, Tramper J, Luyben KCHAM, Klapwijk A (1983) Characterization of *Nitroso on s europaea* immobilized in calcium alginate. Enzyme Microb Technol 5:297 303

Gupta SK, Raja SM, Gupta AB (1994) Simultaneous nitrificationdenitrification in a rotating biological contactor. Environ Technol 15:145-153

Hooijmans CM, Geraats SGM, Van Niel EWJ, Robertson LA (1990a) Determination of growth and coupled nitrification/ denitrification by immobilized *Thiosphaera pantotropha* using measurements and modeling of oxygen profiles. Biotechnol Bioeng 36:931-939

Hooijmans CM, Ras C, Luyben KCHAM (1990b) Determination of oxygen profiles in biocatalyst particles by means of a combined polarographic oxygen microsensor: measurements and modeling of oxygen profiles. Enzyme Microb Technol 12:178-183

Hunik JH, Bos CG, Van Den Hoogen P, De Gooijer CD, Tramper J (1994) Co-immobilized *Nitrosomonas euroapaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in K-carrageenan gel beads. Biotechnol Bioeng 43:1153-1163

Kokufuta E, Shimoshashi M, Nakamura I. (1987) Continuous column denitrification using polyelectrolyte complex-entrapped *Paracoccus denitrificans* cells. J Ferment Technol 65:359-361

Kurosawa H, Tanaka H (1990) Advances in immobilized cell culture: development of a co-immobilized mixed culture system of aerobic and anaerobic microorganisms. Process Biochem Int 25:189-196

Nilsson 1, Ohlson S (1982) Columnar denitrification of water by immobilized cells. Appl Microbiol Biotechnol 14:86-90

Nilsson 1,Ohlson S, Haggstrom L, Molin N, Mosbach K (1980). Denitrification of water using immobilized *Pseudomonas denitrificans* cells. Eur J Appl Microbiol Biotechnol 10:261-274

Robertson LA, Kuenen JG (1990) Combined heterotrophic nitrification and aerobic denitrification in *Thiosphaera pantotropha and* other bacteria. Antonie van Leeuwenhoek 57:139-152

Robertson LA, Corneline R, De Vos P, Radioetomo R, Kuenen JG (1989) Aerobic denitrification in various heterotrophic nitrifiers. Antonie van Leeuwenhoek 56:289-299

Martins dos Santos VAP, Tramper J, Wijffels RH, Tramper J (1992) Integrated nitrification and denitrification with immobilized microorganisms. In: Melo LF *et al* (eds) Biofilms Science and technology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 449 -453

Martins dos Santos VAP, Tramper J, Wijffels RH (t993) Simultaneous nitrification and denitrification using immobilized microorganisms. Biomater Artif. Cells Immobilization Biotech 21:317-322

Martins dos Santos VAPM dos, Marchal JL, Tramper J, Wijffels RH (1996) Modeling and evaluation of an integrated nitrogen removal with co-immobilized microorganisms in double-layer gel beads. Biotechnol. Prog. 12:163-171

Schlegel HG (1986) . *General microbiology.* Cambridge University Press, Cambridge

Tijhuis L (1994) Biofilm airlift suspension reactor: biofilm formation, detachement and heterogeneity. PhD thesis, TU Delft, The Netherlands

Tramper J (1984) Nitrification and denitrification by immobilized bacteria. In: Proceedings of the 3rd European Congress on Biotechnology, VCH Verlag, Weinheim, vol IV, pp 363 368

Tramper J (1987) Nitrification and denitrification by immobilized viable cells. In: Laskin Al *et al.* (eds) Enzyme Engineering vol 8. New York Academy of Sciences, New York, pp 362 366 Van Niel EWJ (I 991) Nitrification by heterotrophic denitrifiers and its relation to autotrophic nitrification. PhD Thesis, TU Delft, The Netherlands

Varseveld HG van (1979) Environmental factors on the efficiency of energy conservation in *Paracoccus deniitrificans.* PhD Thesis, Free University of Amsterdam, The Netherlands Vishniac W, Santer M (1957) The thiobacilli. Bacteriol Rev 21:195-213

Wijffels RH, Tramper J (1989) Performance of growing *Nitrosomonas europaea* cells immobilized in K-carrageenan, Appl Microbiol Biotechnol 32:108-112

Wijffels RH, Schukking A, Tramper J (1990) Characterization of a denitrifying bacterium immobilized in i -carrageenan, Appl Microbiol Biotechnol 34:399-403

Wijffels RH, de Gooijer CD, Kortekaas S, Tramper J (1991) Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in -carrageenan. Part 2. Model evaluation. Biotechnol Bioeng. 38:232-240

Wijffels RH, de Gooijer CD, Schepers AW, Beuling EE, Mall&e LF, Tramper J (1995) Dynamic modelling of immobilized *Nitrosomonas europaea:* implementation of diffusion limitation over expanding micro-colonies. Enzyme Microb Technol 17:462-471

This chapter has been published in: Biotechnology Annual Review, 1996, 3: 227-244 by Martins dos Santos VAP, Vasilevska T, Kajuk B, Tramper J, Wijffels RH.

## CHAPTER 4

# Production and Characterization of Double-Layer Beads for Co-Immobilization of Microbial Cells


## ABSTRACT

The use of double-layer beads can provide a way to immobilize selectively different microbial populations having complementary metabolic pathways, but that would compete for a common substrate if simply co-immobilized as a mixed culture. In this work, several methods are presented to produce double-layer beads for the selective co-immobilization of microbial cells. Coating, encapsulation and bead formation by a double-needle device were studied. Combinations of natural support materials such as carrageenan, alginate and chitosan, synthetic polymers such as polyvinyl alcohol and polyacrylonitril, and pre-formed sintered glass, silica and celite beads, were investigated. The beads produced were evaluated with respect to their mechanical strength, ease of production, shape and mildness for cell immobilization. Beads having synthetic polymers in their outer layers were the strongest of the biocatalytic particles produced. However, their application to immobilization is limited because neither nitrifying nor denitrifying cells immobilized in these supports show any conversion activity. From the double-layer beads tested, alginate/carrageenan beads produced by encapsulation fulfilled the best our requirements.

## INTRODUCTION

In the last few years, much effort has been put into the development of methods to produce multiple-layer biocatalytic supports. In most cases, this has been done to prevent or reduce leakage of growing cells from the supports. Examples of this are the use of two-layer beads in the sparkling wine industries[1-3], beer production[4], denitrification of water[5], and in cream and milk fermentation[6,7]. However, to our knowledge, no studies on the utilization of both layers for cell immobilization have been done yet. The use of double-layer beads would provide a way to immobilize selectively different microbial populations with complementary metabolic pathways. Physical separation of the cell populations would minimize the competition problems often associated with the co-immobilization of microorganisms having similar oxygen demands[8-10]. In an attempt to exploit these potentialities, we have proposed a system for the integration of oxidative and reductive processes within double-layer beads using co-immobilized microorganisms[11]. As an example, we have shown that, by making use of the simultaneous coexistence of aerobic and anoxic zones within the support, coupled nitrification and denitrification could be accomplished in an air-lift reactor with nitrifying and denitrifying cells co-immobilized in separate layers of double-layer beads. Physical separation of the two cell populations avoids that the fast-growing facultative-aerobic denitrifiers would outcompete the slow-growing aerobic nitrifiers.

In this work, we describe several techniques and materials for co-immobilization of microbial cells in double-layer beads. Coating, encapsulation and bead formation using a double-needle device were studied. With these techniques, several combinations of natural gels and synthetic polymers were used both for the envelope and core of the beads. Pre-formed inorganic particles were used as core-beads as well.

For their application in actual reactor systems, double-layer beads should have a good mechanical stability and the cells of interest must be able to grow in them. Furthermore, as we intend to further characterize and model the system above described, we require that both envelope and core should be spherical, the bead production should be reproducible and the beads should be suitable for operation in an air-lift reactor. Hence, based on these requirements, we screened and compared double-layer beads to each other with respect to their mechanical strength, ease of production, reproducibility, shape and mildness for cell immobilization. As a reference, single alginate and carrageenan beads were used. Most of the double-beads tested did not meet simultaneously all these criteria, although several combinations of techniques and materials have shown to result in beads that were either strong, easy to make, reproducible or mild for immobilization of microbial cells. From all the double-layer

beads tested alginate/carrageenan beads produced by encapsulation fulfilled the best our requirements.

## MATERIALS AND METHODS

### Immobilization techniques

Three main techniques for production of double-layer beads were studied: coating, encapsulation and the use of double-needle. A schematic representation of these techniques is shown in Figure 1. In Table I an overview of the immobilization conditions, their range of variation and the materials used is presented. Percentages are expressed in weight per volume (w/v) unless otherwise indicated. To enhance visual contrast between the two layers of the double-beads, Coomassie Brillantblau G250 (Merck) was added to the core beads, except when these were of glass, biolite or celite. All the gels used in the immobilization procedures were maintained at 35°C during the immobilization. Diameters of the inner and outer beads were measured using a stereo microscope (Olympus) with a measuring objective having a graduated scale incorporated. The measuring objective was calibrated using a ruler with mm scale marks. Hundred beads were measured in this way and the average diameters of inner and outer bead as well as the respective standard deviations were calculated.

#### *Immobilization materials*

κ-Carrageenan (Genugel X0828) was obtained from the A/S Kobenhavns Pektinfabrik, Denmark, sodium alginate (Protanal LF 10/60) from Pronova Biopolymers, Drammen, Norway and chitosan (Practical grade) from Sigma, St Louis, MO, USA. Polyvinylalcohol (PVA) (Mol. Wt 70,000-10,000, 11-14 cps) and dextran (Mol. Wt. 60,000 - 90,000) were purchased from Sigma as well. The polyacrylonitril dextran co-polymer (PAN) was a kind gift from Drs. Angelkoska, Kardaleva, Vandeska and Kuzmanova form the Faculty of Technology and Metallurgy of Skopje, Former Yugoslavian Republic of Macedonia[12].

Different types of celite beads (Manville, Denver, Colorado, USA) were used: R-630, ∅=2.7 ± 0.3 mm; R-632, ∅=0.95 ± 0.11 mm and R-633, ∅=0.45 ± 0.08 mm. Siran (sintered glass) beads (diameter 0.5 ± 0.2 mm) were purchased from Schott Nederland, Tiel, The Netherlands. Biolite-F beads (3.1 ± 0.2 mm in diameter) were obtained from Dégremont Holland, Bunnik, The Netherlands.

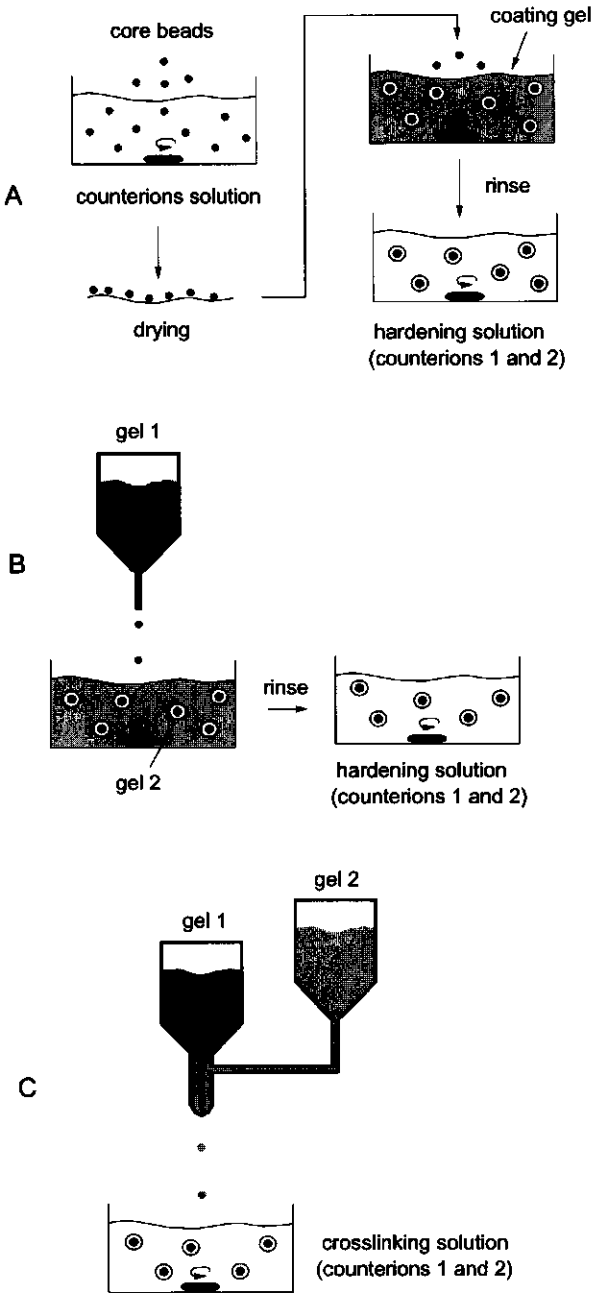


Figure 1 – Schematic representation of the techniques used for the production of double-layer beads: A) coating technique; B) encapsulation; C) double-needle technique.

***Single beads***

Polymeric single beads were prepared by extruding dropwise a polymer solution through a 0.6 mm diameter hollow needle (flat end) into a stirred (400 rpm) solution of their gelling agents (crosslinking/hardening solution, see Table I). The beads were kept stirring for at least 60 minutes in the hardening solution. The PVA droplets were dripped in a saturated boric-acid solution for 15 minutes and then transferred to a phosphate solution (pH 6.5) for hardening as described by Chen & Lin[13]. The immobilization conditions and respective variation ranges for each combination are presented in Table I.

***Coating***

In this procedure, pre-formed core beads were coated by a gel envelope as schematically shown in Figure 1a. The core beads used were both single polymeric beads prepared beforehand as above described, and inorganic solid supports such as celite, biolite and glass beads. The inorganic supports were washed with demineralized water prior to coating.

In the first step, core beads were dropped in a stirred solution containing the gelling agents of the coating polymer. After an immersion time of 60 minutes (for all cases) the beads were transferred to a solution of the coating gel, where they were kept, while stirring, for a given time (varied in the range of 5 to 60 minutes). During this time, the gelling agents present in the core beads diffused outwards and promoted the formation of a gel layer around the core. After that, demineralized water was added (five-fold dilution to prevent agglomeration of beads), the excess of coating gel was decanted and the coated beads were transferred to the hardening solution for at least 60 minutes. The core and coating materials shown in Table I were used in all the combinations.

Since the PAN co-polymer gelates irreversibly merely by contact with water, coating with PAN was simply done by immersion of the wet core beads in the co-polymer solution. Stirring at 300 - 400 rpm was provided by means of a magnetic bar and a Farmo-Geratetechnik M22/l magnetic stirrer.

***Encapsulation***

By this method, double-layer gel beads were produced in a single step as shown in Figure 1b. A gel solution (which will form the inner bead) was extruded dropwise through a hollow needle into a solution of the gel that constituted the external layer of the bead. The inside gel solution contained the counterions of the outside gel and vice-versa. As the inside gel was dropped into the outside gel solution, gelation of both the

inner and outer layers took place. After a given gelation time (see Table I), the external gel solution was diluted five-fold, the beads were collected, rinsed with water and finally placed in a hardening solution containing the counterions of both gels. In all combinations of alginate and carrageenan, KCl 0.75 M and $BaCl_2$ 0.02 M plus $CaCl_2$ 0.08 M were added to the alginate and carrageenan solutions, respectively. The concentration of acetic acid used for preparation of the chitosan solutions was varied in the range 60 - 260 mmol.

***Double-needle***

In this single-step technique (Figure 1c) the polymers were co-extruded dropwise through two concentrically-placed hollow needles into a stirred (400 $min^{-1}$) solution of their gelling agents (Table I). The diameters of the outer and inner needle were 1.0 and 0.5 mm, respectively. The gels were pumped by two Microperrex peristaltic pumps. The proportions between the pumping rates of the inner and outer polymer were varied within the ranges 1:10 and 10:1.

**Assessment of the mechanical stability**

Mechanical stability of the beads was expressed as their resistance to compression. Uniaxial compression of the double-layer gel beads at a constant rate was accomplished with an Overload Dynamics testing instrument (table model S100) fitted with a 2000 N load-cell at room temperature. This apparatus consists of a bottom-fixed plate and a moving bar containing the load-cell. Individual beads were placed on the fixed plate and the moving bar was lowered at a crosshead speed of 3 $mm.min^{-1}$. The force needed for deformation was recorded as function of time until bursting of the beads. Disruption of both the envelope and core was registered. The measurements were done with at least five beads of each type. Single carrageenan, alginate and chitosan beads were submitted to the same compression tests as comparison. The influence of polymer concentration on the mechanical stability of the (single) beads was studied by measuring force applied at fracture for carrageenan, alginate and chitosan concentrations within the range 0.5 - 3% (Table I). A device generating Taylor vortices (two coaxial cylinders where the inner cylinder rotates) was used to assess the resistance of the bead envelope to shear caused by the resulting elongational flow. Shear rates were varied from 136.8 to 811.4 $s^{-1}$ during periods ranging from 5 to 60 minutes. The magnitude of these shear rates is comparable to that of air-lift reactors we commonly use in our research. Batches of 100 capsules were used at a time. The beads were checked visually for rupture of their external layer using a stereo microscope.

**Viability tests**

The ability of nitrifying and denitrifying cells to grow in the immobilization supports was evaluated by measuring the substrate consumption rates ($NH^+_4$ and $NO^-_2$ for nitrifiers and denitrifiers, respectively) both of free cells and of cells immobilized in single beads. Viability of the nitrifying microorganism (*Nitrosomonas europaea* ATCC 19718) was tested for the materials used as envelope (carrageenan, alginate, chitosan, PVA/alginate and PAN), whereas that of the denitrifying cells (*Pseudomonas sp.*, strain LMD 84.60, formerly designated as *Pseudomonas denitrificans*) was evaluated for the core materials (carrageenan, alginate, chitosan, dextran, PVA, celite, biolite and glass beads). Both microorganisms were separately pre-cultivated, collected and concentrated as earlier described[11]. For testing the cell viability in polymeric materials (except PAN), a concentrated cell suspension was divided into several equal portions which were mixed thoroughly with a 30 ml solution of the polymer to be tested (proportion cell suspension:polymer 1:10). Immobilization was done further according to the procedure above described for the production of single beads. The final gel concentration was 2% for the ionotropic gels and 8% for the PVA gel. A 30-ml free-cell suspension to be used as a reference was prepared by mixing one of the concentrated cell portions of each test set with a 0.9% NaCl solution. Each set of beads obtained as well as the free-cell suspension (blank) was added to 200 ml serum flasks containing 80 ml medium (Martins dos Santos et al., 1996) and cultivated in batch at 30°C with gentle agitation. Depending on whether nitrifying or denitrifying cells were tested, aerobic or anaerobic conditions were provided. Substrate conversion was followed in time during 5 to 10 days. All experiments were done in duplicate. Since the PAN co-polymer gelates in contact with water (see above), the cells need to be dried before being mixed with the polymer. Therefore, the viability of the nitrifiers in PAN was evaluated using different drying procedures. A concentrated cell suspension was divided in 5 equal portions. One portion, to be used as a reference, was resuspended in 30 ml of 0.9% NaCl. From the remaining four portions, one was vacuum-dried, two others were freeze-dried, with and without glycerol 5 % (v/v) (cryoprotectant), respectively, and a fourth one was frozen first in liquid nitrogen (-80 °C) and then freeze-dried without glycerol. The four portions of dried bacteria were then added to 4 x 30 ml of the PAN co-polymer. Beads were produced by extruding dropwise the polymer solutions through a 0.6 mm hollow needle into a 0.9% NaCl solution. The four types of beads obtained and the free-cell suspension were then separately cultivated (under aerobic conditions at 30°C) in 5 x 200 ml sterile serum bottles containing 80 ml medium. Ammonia conversion was followed in time during 10 days. The experiments were done in duplicate.

*Table 1.* Overview of the immobilization techniques, materials and immobilization conditions used.

| *Technique* | Core | | Envelope | | | | | Hardening | |
|---|---|---|---|---|---|---|---|---|---|
| | Material | Concentration (% w/v) | Foregoing treatment | | Coating material | Concentration (% w/v) | Gelation time (min) | Agent, concentration | Time (min) |
| | | | Procedure | Gelling agent | | | | | |
| Single beads | alginate<br>κ-carrageenan<br>chitosan | 0.5–3 | – | | – | | 30 | $CaCl_2$, 0.1 M<br>KCl, 0.5 M<br>$KH_2PO_4$, 0.1 M | 60 |
| | PVA | 4–8 | – | | – | | 5–60 | $H_3BO_3$ sat./$Na_3PO_4$, 0.5 M | 15–60 |
| Coating (core beads prepared beforehand) | alginate<br>κ-carrageenan<br>chitosan | 1–3 | immersion in a solution of the gelling agent of the coating gel | KCl, 0.5 M for carrageenan, $CaCl_2$, 0.1 M for alginate, $KH_2PO_4$, 0.1 M for chitosan, $H_3BO_4/KH_2PO_4$, 0.1 M for PVA, $H_2O$ for PAN | Alginate<br>κ-carrageenan<br>Chitosan | 0.3–3 | 5–60 | $CaCl_2$, 0.1 M<br>KCl, 0.5 M<br>$KH_2PO_4$, 0.1 M | 60 |
| | celite | – | | | PVA | 4–8 | | $H_3BO_3$ sat./$KH_3PO_4$, 0.5 M | 15/60 |
| | biolite | | | | PAN | – | 5 | | |
| | glass | | | | | | | | |
| Encapsulation | alginate<br>κ-carrageenan<br>chitosan | 1–4 | mixture with the cross-linking ions of the outer gel | KCl, 0.5 M for carrageenan, $CaCl_2$, 0.1 M+ $BaCl_2$, 0.025 M for alginate $KH_2PO_4$, 0.08 M for chitosan | alginate<br>κ-carrageenan<br>chitosan | 0.15–2 | 5–30 | KCl, 0.5 M + $CaCl_2$, 0.1 M<br>$KH_2PO_4$, 0.1 M | 60 |
| | dextran | 20 | | | | | | | |
| Double-needle | alginate<br>κ-carrageenan<br>chitosan | 0.5–3 | – | | alginate<br>κ-carrageenan<br>chitosan | 0.5–3 | 30 | $CaCl_2$, 0.1 M<br>KCl, 0.5 M<br>$KH_2PO_4$, 0.1 M | 60 |
| | PVA | 4–8 | | | PVA | 4–8 | | $H_3BO_3$ sat./$KH_3PO_4$, 0.5 M | 15/60 |
| | dextran | 20 | | | | | | | |

The viability of the dried cells alone was tested by resuspending them in 30 ml saline and cultivating them as above.

The ability of the denitrifying cells to grow in the inorganic carriers was tested also by measuring the nitrite conversion during anaerobic batch cultivation of the cells immobilized in each type of support. Both a free-cell suspension and cells immobilized in carrageenan were used as a reference. Before being used for immobilization, Biolite-F, Celite R-633 and Siran 041 beads were washed abundantly with demineralized water. About 12 ml beads of each material were packed in a 20-ml syringe barrel (Plastipak, i.d. 19 mm). Hundred ml of a concentrated (0.8 $g_{dry\ weight}.l^{-1}_{suspension}$) *Pseudomonas* spp. suspension were continuously circulated through the column for 60 minutes at a flow rate of 60 $ml.min^{-1}$ using a Watson-Marlow 502S peristaltic pump (Watson-Marlow Ltd., Falmouth, England). The columns were then washed with 10-column volumes buffer to remove biomass present in extra-particle fluid. Immobilized cells were cultivated in batch, at 30°C, by recirculating continuously 100 ml medium[11] through the reactor at a rate of 10 $ml.min^{-1}$. Helium at a flow rate of 50 $ml.min^{-1}$ was bubbled through the column to maintain the system anaerobic. Liquid samples of the reactor outlet were taken every ten or twenty minutes. The results were corrected for the volume withdrawn for sampling. Immobilization in the porous supports and in κ-carrageenan was done using a cell suspension five-fold more concentrated than that of free cells used as reference.

**Analytical methods**

Ammonia, nitrite and nitrate concentrations in batch media and in the influent and effluent of the reactors were measured as described elsewhere[14].

## RESULTS

The beads obtained were divided in three main groups as produced respectively by coating, encapsulation and by a double-needle device (Table 1).

**Qualitative aspects**

***Single beads*** - Polymeric single beads with diameters between 1 and 2.5 mm were easily produced within the concentration ranges tested. Such beads are used routinely and have been described in literature [14-16]. In the present work they are used mainly as core materials for the production of double-layer beads. For that reason the production of single beads is not further described.

***Coating*** - Spherical double-layer particles were obtained using both polymeric materials and inorganic solid particles as core in all combinations tested (Table 1). The beads obtained had a core and envelope that were physically separated from each other, as could be observed by examining the beads with a microscope (see also *Encapsulation*) and by "squeezing" them between two fingers.

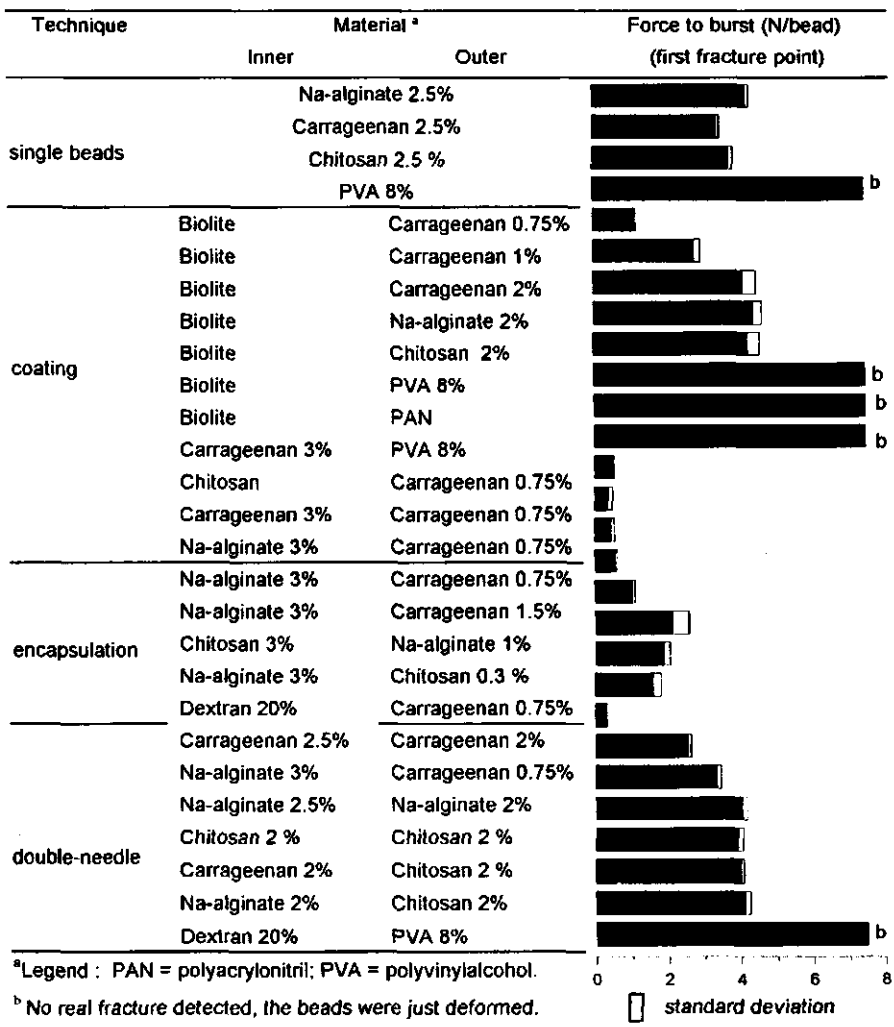


Figure 2 – Overview of the double-layered beads produced.

When ionotropic polymeric materials were used as core, their gel concentration had to be at least twice as high of that of the coating gels (alginate, carrageenan and chitosan). For smaller concentration differences the core beads dropped into the solution could not get through the coating-gel surface, independently of the stirring rate or dropping height. In practice, this meant that the gel concentration of the ionotropic coating-solution did not exceed 1.3 %. This limitation did not apply to the inorganic core materials, but for coating-gel concentrations higher than 2% it was difficult to produce double-layer beads because the solution was too viscous. Coating of carrageenan, glass, biolite and celite beads with PVA and PAN gels was successful. Due to the high viscosity, however, extensive formation of clumps occurred especially at higher gel concentrations (typically more than 1% for the ionotropic gels). In all cases, the fraction of clumps of beads increased with the increase of coating-gel concentration. For coating times of less than 5 minutes, very thin and weak external layers were formed, whereas for times of 25 minutes or more, no further increase in the envelope thickness (and thus strength, see Mechanical properties) could be observed. However, the fraction of clustered beads increased (up to 50%) with the coating times in all cases. Optimal times (with respect to these two factors) between 10 and 15 minutes were found, which was reflected in envelope thickness of about 0.8 to 1.5 mm after hardening.

***Encapsulation*** - In all beads produced by encapsulation, both the core and envelope had a very regular spherical shape as shown in Figure 2. A concentration difference of at least a factor two between the core and envelope gels was required to obtain double-layer capsules. Again, the gelation time was important and for times of less than 3 minutes, a weak and very thin envelope was formed whereas for times longer than 20 minutes, the capsules began to loose their spherical shape. Thickness of the external layer also increased with the envelope gel concentration, but for a gel of more than 1.8%, the viscosity made capsule formation very difficult. For a gelation time of 15 minutes, capsules of 3% alginate (core) and 1.5% carrageenan (envelope) with a total diameter of about 5 mm and core diameter of about 3 mm were obtained, with a fraction of clustered beads of about 20%. Except for the mechanical strength (see Assessment of the mechanical stability ) the capsules produced by the different combinations shown in Table 1 were very similar.

***Double-needle technique*** - This one-step method was the simplest to apply as the double-layer gel droplets were just pumped through a hollow double-needle into a stirred solution of their gelling agents. There was thus no formation of clusters nor

limitations in the outer and inner gel concentrations with respect to each other, although for concentrations above the 3.5 - 4 % the viscosity of the gels made the flow through the needles very difficult. Furthermore, no gap could be observed between the two layers nor was it possible to clearly separate the core and envelope. The double-layer beads obtained formed a continuous solid phase, even if different gels were used as outer and inner matrices. The core was always located within the (externally) spherical capsule. In all cases, however, the shape of the core was very irregular and there appeared to exist a certain degree of mixing between the two layers. Variation of the ratio between the flows of the inner and outer gel (within the ratio 0.1 to 10) had no visible positive influence on this, neither had variations in the concentration of the gels (by which their viscosity and density changed).

**Mechanical stability**

Mechanical stability of the beads was expressed as their resistance to compression. A typical result of a uniaxial-compression measurement (3 $mm.min^{-1}$) is shown in Figure 3 for a double-layer 3% alginate (core) and 1.5% carrageenan (envelope) bead obtained by encapsulation.

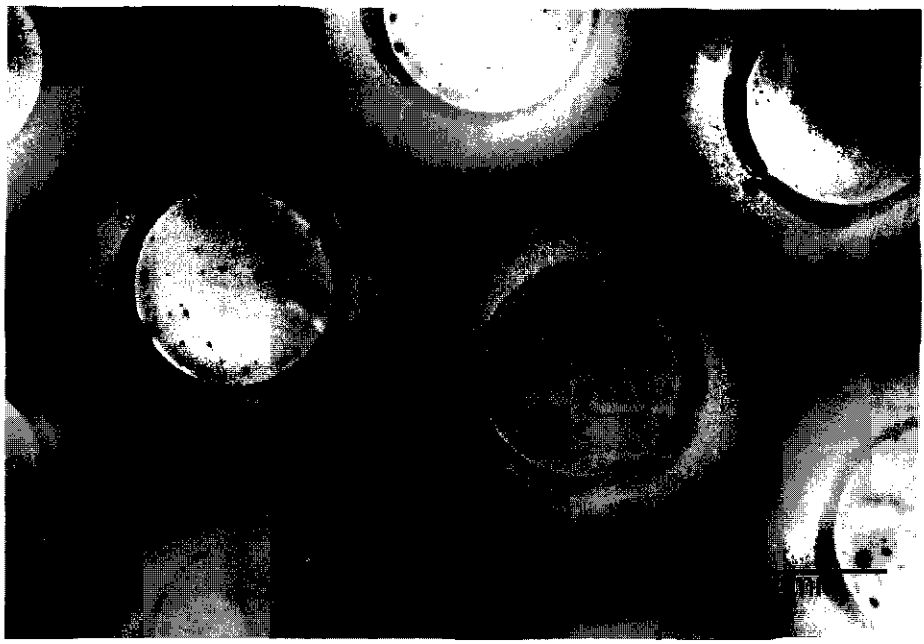

Figure 3 – Double-layer beads of 3% (w/v) alginate and 1.5% (w/v) κ-carrageenan produced by encapsulation. Diameter (mm): core = 2.4 ± 0.1; total = 6.4 ± 0.1.

The two peaks observed correspond to the disruption of the outer layer first, followed later by (macro)fracture of the inner bead. As the force needed for disruption of the bead or layer increases as a function of its size (results not included), the data presented in Figure 4 and Table 2 refer, as much as possible, to beads with approximately the same core and total bead size (3 and 5 mm in diameter, respectively).

The influence of the ionotropic-polymer concentration on the mechanical stability of (single) beads was studied by measuring their resistance to compression for gel concentrations varying in the range 0.5 - 3%. For the three polymers tested, the gel strength was of the same order of magnitude and increased more or less linearly with the polymer concentration (Figure 4). This tendency agrees with that shown by Martinsen *et al.* [17] for alginate alone.

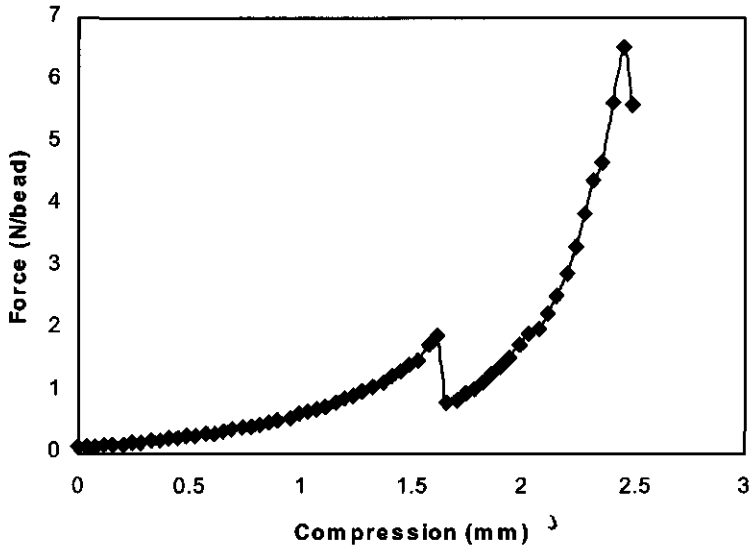


Figure 4 – Typical result of an uniaxial compression measurement of a double-layer gel bead.

In the last column of the table in Figure 2 the force necessary to burst the envelope of double-layer beads is shown. The force needed to disrupt beads of carrageenan, alginate and chitosan at concentrations earlier used in immobilized-cell studies is given as a reference[5,18,19]. Since the behaviour of coated biolite, celite and glass beads was very similar in all the combinations, only coated biolite beads are represented in the figure.

Beads coated by PVA or PAN were very resistant to compression and in most cases just (extreme) deformation was observed, also for carrageenan beads coated with PVA. Also, double-layer beads produced by the double-needle technique in which the outer layer was made of PVA or PAN could be only deformed and not disrupted. The coatings of the inorganic core beads were stronger than single beads of the same (natural) gel material at the same concentration. Examples of this are glass beads coated with 2% alginate (12.8 and 8.5 N for the double and single beads, respectively). The coatings made of alginate, carrageenan or chitosan were much weaker if the core beads were made of these natural gels than if the cores were inorganic. An increase in the gel concentration in the coating-gel solution resulted always in stronger beads, similarly to what was observed for single gel beads.

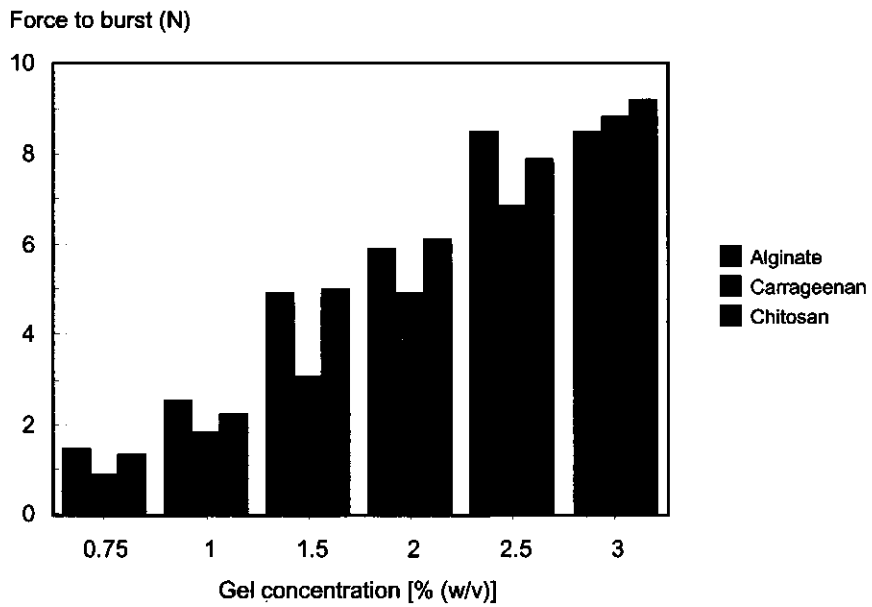


Figure 5 – Force to burst (constant force) single beads of alginate, carrageenan and chitosan as a function of the gel concentration measured at constant force.

In spite of having a liquid core, capsules of chitosan (core) and alginate (envelope), and chitosan and carrageenan had a resistance to compression comparable to that of 3% alginate - 1.5% carrageenan beads. Chitosan is a polycation and it crosslinks polyanions such as alginate and carrageenan. This enhances the mechanical resistance of the capsules as suggested by Tay *et al.*[19]. As a comparison, capsules of 20% dextran (liquid)

and 0.75% carrageenan were tested. These were indeed significantly weaker (0.9 N) than capsules of 3% chitosan (liquid) and 0.75% carrageenan (3.1 N). Beads produced by encapsulation were in general stronger than those produced by coating for the same gel combinations, as illustrated by the combination alginate 3% (core) carrageenan 0.75% (outer layer) (2.1 N and 1 N, respectively). The force at fracture of the double-layer beads of the same gel made by the double-needle technique were about the same as those for the single beads at the same concentration. No gap could be observed between them nor was it possible to clearly separate the layers as the double-layer beads seemed to form a continuous solid phase. To check the importance of the interaction between the layers, double-layer beads of 3% carrageenan plus 0.75% carrageenan, and 3% of alginate plus 0.75% carrageenan were produced and tested for fracture. The beads made by the double-needle technique were two and four times stronger than those produced by encapsulation and coating, respectively.

In almost none of the cases a significant rupture of the gel coatings in the shear experiments with a device generating Taylor vortices was detected. The shear rates attained (up to 811.4 $s^{-1}$) were of the same order of magnitude (400 - 900 $s^{-1}$) as those estimated (using Kolmogoroff's theory of isotropic turbulence, see Kawase & Moo-Young[20]) for a 3-L gas-lift reactor that we commonly use for immobilized-cell studies, but the time scales (5-60 min) were far shorter then those commonly used (days or months).

**Cell viability**

Viability tests based on substrate consumption were accomplished with *Nitrosomonas europaea* and *Pseudomonas* spp. separately immobilized in the various supports. Free cells were used as a reference.

Both nitrifiers and denitrifiers grew well when immobilized in k-carrageenan and alginate (not shown), which agrees with numerous other studies that showed that these bacteria easily maintain their metabolic capacities after immobilization in both alginate and κ-carrageenan[14-16,18]. In contrast, neither the nitrifying nor denitrifying cells were able to convert significantly their respective substrates when immobilized in chitosan or PVA. Nitrifying cells also did not seem to survive the (freeze)drying processes applied, even in the presence of glycerol as cryoprotectant. Ammonia was neither converted by any of the dried-cell suspensions nor by the nitrifying dried-cells immobilized in PAN. The cell activities of the reference cell-suspensions were 3.2 and 2.5 $mmol.l^{-1}.d^{-1}$, respectively, for the tests with dried-cell suspensions and immobilized dried cells.

Denitrifying cells immobilized in celite, biolite and glass beads were cultivated in batch using both a free-cell suspension and cells immobilized in carrageenan as a reference. In all cases, immobilized cells converted nitrite at relatively high rates (Figure 5). The differences among them were probably due to differences in the immobilization efficiency. Immobilization of microbial cells in solid porous supports has been earlier reported in literature[22-24].

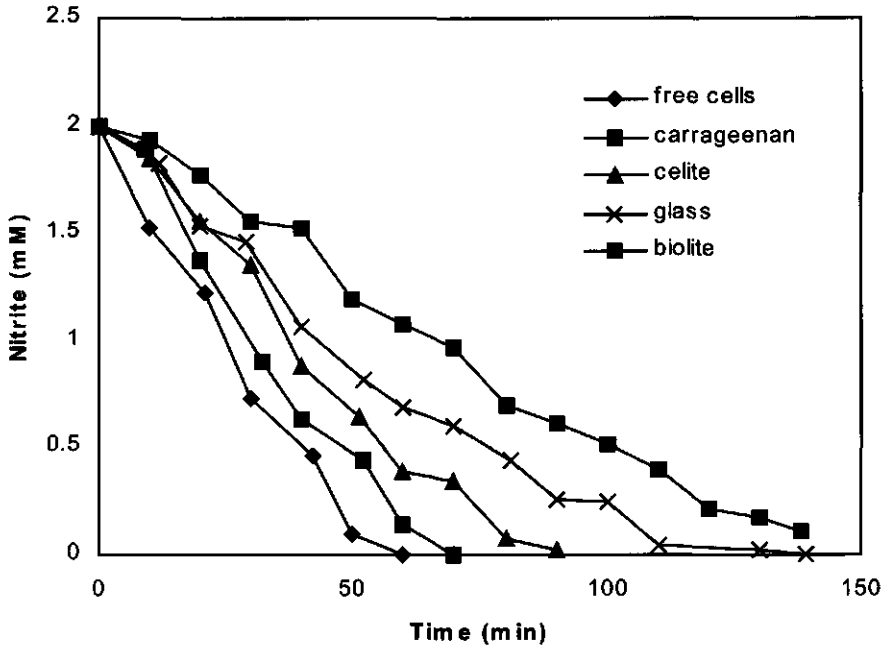


Figure 6 –Time course for nitrite reduction with cells in suspension and cells immobilized in κ-carrageenan, celite, glass and biolite beads.

## DISCUSSION

### Comparison of the three techniques

A potential merit of the coating procedure is that beads of very different nature can be used both as core material and as coating. A wide range of combinations can be found, as shown by the first group in Table 2. Although the procedure is relatively complicated and difficult to regulate and scale-up, it can be optimized and standardized, as done by Yamagiwa *et al.*[24], who extensively studied the kinetics of coat formation of alginate two-layer beads. From Table 2, it can be concluded that the mechanical strength of the outer layer is determined not only by the coating material but also, to a large extent, by the core itself. The ionotropic coating of double-layer beads having an inorganic hard

core withstood a far larger pressure than those having a gel core. This is because in double-layer gel beads, both the core and coating were deformed upon compression, whereas in those with an inorganic core just the coating could deform. This means that complete-gel beads deformed more than those with inorganic cores at the same compression applied and thus, locally, higher stresses were attained. This resulted in less resistance to compression of the total-gel bead than that of beads with hard cores.

The production process of encapsulated beads is in itself much simpler than the coating procedure since it is done in one step. There are therefore less problems of formation of clusters and in regulation of the production process than with the coating procedure. The beads produced also had stronger outer layers than those of the same composition made by coating, probably because both layers are still liquid when they come in contact with each other, which enhances the diffusion of the counterions through the layers. This is likely to lead to an increase in the local gel concentration and thus to enhanced mechanical strength. Skjåk-Bræk *et al.*[25] showed that the relative diffusion rates of ions and respective polymer govern the gel-formation process and that by regulating these, beads of different degrees of (in)homogeneity in the actual gel concentration and strength are obtained. The encapsulation method is however less flexible than coating as it demands compatible gels (i.e., gels that are not affected by each other's gelling agents) and it is somewhat limited by the necessary density difference and viscosity between the inner and outer gels.

Beads made by the double-needle technique were the easiest to produce, in shorter time, at mild conditions and with less loss of immobilization materials and biomass. Furthermore, these beads were mechanically very stable. A wide range of polymers can be used with this technique, providing that the viscosity does not prevent flow through a nozzle or needle. The shape of the inner beads was, however, very irregular for all polymer combinations and the (certain degree of) mixing between the two layers prevents a complete separation of co-immobilized populations. This is very likely to occur if the boundary tension between the internal and external gel solutions is very low. To verify this, both olive oil (Log P ~ 24.1, density 0.92 at 25°C ) and FC-40 (Log P = 11.4, density 1.87 at 25°C ) were used as inner solution to prepare double-layer beads with 2% carrageenan in the outer layer (hydrophilic, density 1.08 at 25°C). For both cases, and for all flow-rate ratios tested, the inner "bead" was spherical and the two layers were clearly separated from each other, which supports our hypothesis. Since the two layers are inmiscible, a boundary surface between the inner and outer layers will exist, resulting thereby in an increased boundary tension between the core and envelope. The use of apolar solutions as support for immobilization is however limited and

therefore we excluded the beads produced by the double-needle technique from further analysis.

Both the coating and encapsulation techniques are suitable for the production of double-layer beads. To our purposes, yet, double-layer beads of alginate (3%) and carrageenan (0.75%) produced by encapsulation appeared to be the most suitable because, though not being the strongest beads, they were strong enough to be operated in a small-scale air-lift reactor, they could be made in a reproducible way and both layers had a very regular spherical shape. Moreover, since both alginate and carrageenan are relatively soft gels, experimental determination of biomass and solute profiles throughout the beads is facilitated, which is important for model validation in immobilized-cell studies. Application of cells co-immobilized in the two layers of such beads has been reported elsewhere[11,26].

**Viability**

The high acidity of the chitosan matrix (pH 2.5) most likely accounted for the non-survival of both nitrifying and denitrifying cells. For this reason, we excluded chitosan as immobilization material. Nevertheless, some successful applications of cells immobilized in chitosan have been reported for other cells systems[27-29].

Polymerization of PVA (by the method used here) requires boric acid and this seemed also to be lethal to the cells even for short treatment times (10 minutes). Yet, continuous cultivation of denitrifying sludge entrapped in phosphorylated PVA has been successfully reported[13], as well as immobilization of different cell-types in PVA polymerized by freeze-drying[30], freeze-thawing[31-33] and in boric acid alone[34]. In our research and for all beads involving PVA, neither nitrifying nor denitrifying cells were active after immobilization. We are at present further investigating the viability of nitrifying and denitrifying cells in PVA.

## CONCLUSIONS

A very versatile range of applications for (co-)immobilization of microbial cells is possible due to the numerous combinations of materials and techniques available. The choice of the most suitable combination will depend on the specific requirements of the user, namely the mechanical stability, the shape of the beads/layers, ease of production or mildness for cell immobilization. Some of the materials, such as synthetic polymers or chitosan, require harsher immobilization conditions by which the viability of the cells may be critically reduced. Especially this last aspect surely demands further research because their high mechanical stability make these materials particularly adequate for

their use in reactor systems (e.g, in wastewater treatment plants). Other synthetic materials (such as PEG, see Tanaka *et al.*[35] or PCS, see Muscat *et al.*[36]) have been recently reported to combine excellent mechanical properties with high cell viability.

## ACKNOWLEDGMENTS

We thank Katja Grolle and Dr. Ton van Vliet from the Group of Food Physics, Department of Food Science, Wageningen Agricultural University, for excellent technical assistance with the rheological tests and for helpful discussions, respectively. Financial support by J.N.I.C.T., Portugal, is gratefully acknowledged as well (grant BD/3753/94).

## REFERENCES

1. Godia F., Casas C. and Sola C. Application of immobilized yeast cells to sparkling wine fermentation. *Biotechnol.Prog.* 1989, **7**, 468-470
2. Yamagiwa K., Shimizu Y., Kozawa T., Onoder M. and Ohkawa A. Ethanol production by encapsulated and immobilized yeast. *Biotechnol. Techn.* 1994, **4**, 271-74
3. Digat B. A new bioencapsulation technology for microbial inoculants. *Biomat., Art. Cells & Immob. Biotechnol.* 1993, **21**, 299-306
4. Masschelein C. and Ryder M. Beer production with immobilized yeast. Abst. book of Bioencapsulation III - the reality of a new industrial tool. 1993, 20-23 October, Brussel, Belgium, pp. 5-11
5. Klein J., Vorlop K-D. and Steinert H-J. 1985. European Patent nr 85110496.8
6. Prevost H. and Divies C. Cream fermentation by a mixed culture of *Lactococci* entrapped in two-layer calcium alginate beads. *Biotechnol. Letters.* 1992, **14**, 583-588
7. Champagne C.P., Gaudy C., Poncelet D. and Neufeld R. *Lactococcus lactis* release from calcium alginate beads. *Appl. Environ. Microbiol.* 1992. **58**, 1429-1434
8. Kurosawa H., Nomura N. and Tanaka H. Ethanol production from starch by a co-immobilized mixed culture system of *Aspergillus awamori* and *Saccharomyces cerevisiae. Biotechnol. Bioeng.* 1989. **33**, 716-723
9. Kurosawa H. and Tanaka H. Advances in immobilized cell culture:development of a co-immobilized system of aerobic and anaerobic microorganisms. *Process Biochemistry International.* 1990. **25**, 189-196.
10. O'Reilly A.M. and Scott J.A. Defined coimmobilization of mixed microorganism cultures. *Enzyme Microbial Technol.* 1995, **17**, 636-646
11. Martins dos Santos V.A.P.M., Tramper J. and Wijffels R.H. The Magic Bead concept: an integrated approach for nitrogen removal. *Appl. Microbiol. Biotechnol.* 1996, **45**, 447-453

12. Angelkoska, N. Kardaleva., O., Vandeska, E. and Kuzmanova, S. Polyacrilonitryl-dextran co-polymer as a novel support for immobilization of cells. Proceedings of the 7th European Congress on Biotechnology, Nice, France. 1995. Abstract MAP 89.
13. Chen K-C.and Lin Y-F. Immobilization of microorganisms with phosphorylated polyvinyl alcohol (PVA) gel. *Enz. Microbial Technol.* 1994. **16,** 79-83
14. Wijffels R.H., Schukking A. and Tramper J. Characterization of a denitrifying bacterium immobilized in k- carrageenan. *Appl. Microbiol. Biotechnol.* 1989, **34,** 399-403
15. Wijffels R.H., De Gooijer C.D., Kortekaas S. and Tramper J. Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in κ-carrageenan. Part 2: Model evaluation. *Biotechnol. Bioeng.* 1991. **38,** 232--240
16. Nilsson I. and S. Ohlson. Columnar denitrification of water by immobilized *Pseudomonas denitrificans* cells. *Eur. J. Appl. Microb. Biotechnol.* 1982, **14,** 86-90.
17. Martinsen A., Skjåk Bræk G. and Smidsrod O. Alginate as immobilization material. *Biotechol. Bioeng.* 1969, **33,** 79-89
18. van Ginkel C.G., Tramper J., Luyben K.Ch.A.M. and Klapwijk A. Characterization of *Nitrosomonas europaea* immobilized in calcium alginate. *Enzyme Microbial Technol.* **5**:297-303
19. Tay L-F., Khoh L-K., Loh C-S. and Khor E. Alginate-chitosan coacervation in production of artificial seeds. *Biotechnol. Bioeng.* 1993. **42**:449-454
20. Kawase Y. and Moo-Young M. Mathematical models for design of bioreactors: applications of Kolmogoroff's theory of isotropic turbulence. *Chem. Eng. J.* 1990. **43**:B19-B41
21. Salter G.J., Kell D.B., Adams L.A., Brown A.J. and James R. Hydrodynamic deposition: a novel method of cell immobilization. *Enz. Microbiol. Technol.* 1990. **12**:419-430
22. de Backer L. and Baron G. Immobilization of yeast cells in spherical porous glass particles: consequences of intraparticle convection. 1993. Procs. 6th European Congr. Biotechnol. 493-496
23. Aivasidis, A. Process development for anaerobic wastewater treatment using porous carriers for microbial colonization. *Bioprocess Eng.*1989, **4,** 19-25
24. Yamagiwa K., Shimizu Y., Kozawa T., Onoder M. and Ohkawa A. Formation of calcium-alginate gel coating on biocatalyst immobilization carrier. *J. Chem. Eng. Japan.* 1992, **25,** 723-728
25. Skjåk-Bræk G., Grasdale H. and Smidsrod O. Inhomogeneous polysacharide ionic gels. Carbohydrate Ploymers. 1989, **10,** 31-54.
26. Martins dos Santos V.A.P.M., Marchal, J. L., Tramper J. and Wijffels R.H. Modeling and evaluation of an integrated nitrogen removal with co-immobilized micro-organisms in double-layer gel beads. *Biotechnol. Prog.* 1996, **12**, 240-248
27. Knorr D., Pandya Y., Bumont M.D. and Ullrich S. Immobilization of plant cell cultures in complex coacervate capsules for secondary metabolite production. In: Engineering and

Food, Vol 3, Advanced Processes. Spiess WE & Schubert H (Eds). Elsevier Science, London, UK, 1990, pp. 516-523

28. Vorlop K.D. and Klein K. Formation of spherical chitosan biocatalysts by ionotropic gelation. *Biotechnol. Letters*, 1981, **3**, 9-14.
29. Groboillot A.F., Champagne C.P., Darling G.D., Poncelet D., and Neufeld R.J. Membrane formation by interfacial cross-linking of chitosan for microencapsulation of *Lactococcus lactis. Biotechnol. Bioeng.* 1993, **42**,1157-1163
30. Shindo S. and Kamikura M. Immobilization of yeast with hollow PVA gel beads. *J. Ferm. Bioeng.* 1990, **70**:232-234
31. Ariga O., Takagi H., Nishizawa H. and Sano Y. Immobilization of microorganisms with PVA hardened by iterative freezing and thawing. *J. Ferm. Technol.* 1987, **65**, 651-658
32. Asano, H., Myoga, H., Asano, M. and Toyao M. Nitrification treatability of whole microorganisms immobilized by the PVA freezing method. *Water Sci. Technol.*1992, **26**, 2397-2400
33. Myoga H, Asano H, Nomura Y, Yoshida Y (1991) Effects of immobilization conditions on the nitrification tretability of entrapped cell reactors using the PVA freezing method. Wat. Sci. Tech. 23:1117-1124
34. Hashimoto S. and Furukawa K. Immobilization of activated sludge by PVA-boric acid method. *Biotechnol. Bioeng.* 1987, **30**, 52-59
35. Tanaka K., Tada M., Kimata T., Harada S., Fujii Y., Mizuguchi T., Mori N. and Emori H. Development of a new nitrogen removal system using nitrifying bacteria immobilized in synthetic resin pellets. *Wat. Sci. Technol.* 1991, **23**, 681-690
36. Muscat A., Beyerdorf J. and Vorlop K-D. Polycarbamoylsulphonate hydrogel, a new polymer materila for cell entrapment. *Biosens. Bioelectronics* 1995, **10**, 11-14

This chapter has been published in: Biotechnology & Bioengineering, 1997, 56: 217-229 by Martins dos Santos VAP, Leenen EJTM, Ripoll-Martinez M, Van der Sluis C, van Vliet T, Tramper J, Wijffels RH.

## CHAPTER 5

# Relevance of Rheological Properties of Gel Beads for Their Mechanical Stability in Bioreactors


### ABSTRACT

The mechanical stability of biocatalyst particles in bioreactors is of crucial importance for applications of immobilized-cell technology in bioconversions. The common methods for evaluation of the strength of polymer beads (mostly force-to-fracture or tensile tests) are, however, not yet proven to be relevant for the assessment of their mechanical stability in bioreactors. Therefore, we tested fracture properties of gel materials and investigated their relevance for abrasion in bioreactors. Abrasion of gel beads was assumed to be a continuous fracturing of the bead surface. At first three rheological properties were considered: stress at fracture, strain at fracture and the total fracture energy. If stress at fracture is most important, beads having a similar fracture energy but with a smaller stress at fracture would abrade faster in a bioreactor than beads with a larger stress at fracture; if fracture energy is determining, beads that require less energy to fracture would abrade faster than those having a larger fracture energy for the same fracture stress. Therefore, beads of κ-carrageenan and agar (at two different polymer concentrations) were tested for abrasion in four identical bubble columns under the same operating conditions. We expected agar beads to abrade faster than those of carrageenan because agar had either a lower stress at fracture or a lower fracture energy. However, no correlation between fracture properties and abrasion rate was found in any of the combinations tested. Carrageenan beads abraded faster than those of agar in all combinations. Furthermore, both the stress and strain at fracture of agar and carrageenan beads decreased during the run and those of carrageenan decreased faster suggesting that the gels are liable to fatigue in different ways. This hypothesis was confirmed by oscillating experiments in which gel samples were subjected to repeated compressions below their fracture levels. Their resistance to compression clearly decreased with the number of oscillations. Fatigue is probably related to the development of microcracks and microfracture propagation within the material. We concluded that a) the use of tests based on bead rupture do not provide relevant information on the mechanical stability of gel beads to abrasion, and b) abrasion of polymer beads is likely to be related to fatigue of the gel materials.

## INTRODUCTION

Industrial applications of immobilized biocatalysts have been gaining importance in the last decades. Besides their use in pharmaceutical and food biotransformations[43], immobilized-cell processes are promising for wastewater-treatment purposes in general and for nitrogen removal in particular[10,34,44,55]. Numerous studies have shown that high capacities can be reached in air-lift reactors containing (co-)immobilized (de)nitrifying cells[23,34,54]. In some cases, such as that of Hitachi, Japan[41], plants for nitrogen removal based on immobilized-cell technology have been in operation for some years already. For the application of these technologies at full scale in wastewater-treatment processes, the mechanical stability of the support materials is a crucial factor. Indeed, a feasibility study on the application of an immobilized-cell system for nitrification of domestic wastewater has shown that this process can be economically more attractive than conventional activated-sludge systems if the alginate supports remain stable for at least two months[26]. This means that, besides being inert, insoluble in the liquid medium and not biodegradable, support materials for cell immobilization should be mechanically stable under reactor operation[26]. This is, however, mostly not the case both in aerated stirred tanks[5,33,36] and in air-driven reactors[11,19]. In all these reactors, the biocatalytic particles are continuously subjected to hydrodynamic shear stresses, motion and bursting of gas bubbles, and collisions against other particles and reactor parts. In stirred reactors, the energy dissipation is highly localized in the neighbourhood of the stirrer[6,25] whereas in air-driven reactors the shear fields are comparatively more homogeneous[29,30]. This results in local shear stresses that are generally lower than those present in stirred tanks at the same power input[8]. Nevertheless, severe abrasion of biocatalyst particles has been reported to occur in such reactor systems[11,19]. Mechanical stresses can manifest themselves in many ways in a reactor, but the effects of these stresses on a biocatalyst particle largely depend on the mechanical properties of the particle itself. That is to say that they depend not only of the size, shape and density of the particles (properties that are important in the interactions between the particles and the hydrodynamic flows in the reactor), but also of their roughness, hardness, elasticity and degree of homogeneity. These properties determine how a biocatalyst particle is able to accommodate the stresses to which it is subjected in a reactor. Some excellent studies on the chemical and physical properties of polymers for cell immobilization have been done[13,28,37]. However, very little is known about the relevance of these properties for the mechanical stability of cell supports and their resistance to abrasion in (air-driven) bioreactors. In most studies dealing with support materials for cell immobilization, beads are compared with each other on basis of their resistance to compression or tension, but there is no direct

evidence of the relevance of these tests for the mechanical stability of biocatalytic particles in bioreactors, except for applications in packed-bed columns[7,22].

In this work we concentrated on the study of rheological properties that are expected to be relevant with respect to abrasion in air-driven reactors. Several fracture properties were measured for different gel materials. On basis of these properties, different types of gel beads were tested for abrasion in identical bubble columns under the same operating conditions. No correlation was found between the properties measured and the abrasion found. Abrasion appeared to be more related to fatigue of the gel materials than to their initial resistance to macroscopic fracture. Fatigue is likely to be related to the development of microcracks and microfracture propagation within the material.

## THEORY

### *Abrasion mechanisms*

In agitated reactors, either air-driven or stirred, shear stresses are always present. These are due to hydrodynamic interactions (either liquid-bead or wall-bead), motion and bursting of gas bubbles, and collisions against other particles and reactor parts. The possible stresses and interactions will be shortly discussed below. Furthermore, the possible shear stresses that could have been present in the reactors were calculated. For explanation of the symbols see section Nomenclature. All values were estimated for the experimental conditions described in the section Materials and Methods. The average energy dissipation was calculated according to the expression[8]:

$$\langle \varepsilon \rangle = \frac{F_g}{m}\frac{1}{V}RT\ln\left(\frac{P_s}{P_l}\right) \qquad (1)$$

The average collision rates for each of the ranges was calculated assuming isotropy, i.e., that the local energy dissipation rate ($\varepsilon$) is equal to the average one ($\langle\varepsilon\rangle$). In the experiments described $\langle\varepsilon\rangle = 0.54\ W.kg^{-1}$.

*Liquid shear* can be interpreted using the Kolmogoroff theory of isotropic turbulence. According to this theory, the energy transmitted to a reactor (power input) is first transferred to large-scale eddies (moving elements of the fluid containing a certain kinetic energy) which are unstable and break up into smaller eddies, and these into even smaller ones and so on. This irreversible energy transfer occurs within the full range of eddy sizes, until the energy is totally dissipated by viscous forces into heat by the smallest eddies. If particles are present in the fluid, part of this energy may be used for mechanical work (e.g.

rotation of the bead) on the particle surface possibly causing damage to the particle. Indeed, eddies with a size of the same order as the bead cannot engulf the particles and will thus exert a local stress on the beads surface. Thomas[46] has postulated that instantaneous pressure differences across a particle caused by turbulent fluctuations lead to (local) deformation and possibly fracture. Expressions for estimation of the average pressure differences and maximum pressure (stress) acting on a bead are described by Thomas[46], Kawase & Moo-Young[21], Cherry & Papoutsakis[6] and Baldyiga & Bourne[3,4]. Baldyiga & Bourne[3] estimate the average and maximum pressure (stress) difference acting on a bead with the following expressions:

$$< p(d) > \approx C_p \rho (< \varepsilon > d_b)^{\frac{2}{3}} \qquad (2)$$

$$p_{max}(d) = <p(d)> (\frac{d_b}{L})^{-0.587} \qquad (3)$$

For the experiments described here the calculated average (<p(d)>) and maximum ($p_{max}(d)$) pressure difference are then respectively approximately 23 and 176 N/m$^2$.

*Bubble shear*- For air-driven bioreactors, the damaging interaction of gel beads with air bubbles can take place in three different zones of the reactor: sparger zone, where the air bubbles are formed; bulk zone, where the air bubbles rise, and surface zone at the top of the reactor, where air bubbles break up. To roughly estimate the shear stresses a particle could encounter in each of these three different zones the method described by van 't Riet & Tramper[49] can be used. The assumption made in this method is that at one side of the bead the liquid velocity is equal to a calculated value and at the other side zero. Using these expressions only very low shear stresses were calculated (< 1 N/m$^2$).

*Wall shear* - In air-driven reactors the flow is mostly turbulent and liquid elements can have high velocities in the bulk of the reactor. On the reactor surfaces, however, the liquid velocity is zero (non-slip condition) and thus a velocity gradient between the bulk and the surfaces establishes. These gradients exert stresses on the beads that are brought into the vicinity of the walls. If these velocity gradients are high enough, they may damage the beads. An expression used to estimate the average magnitude of these stresses is given by Tramper & Vlak[47]:

$$\tau = 0.5 \rho <v_{id}^2> k_f \qquad (4)$$

and yielded approximately 140 N/m$^2$ for the experiments done.

*Collisions between particles*- As above described, in any agitated reactor, at every moment, there are always eddies in a broad range of sizes. Since these eddies have different velocities (not just in magnitude but also in direction) the particles present in the bulk will be subjected to a large range of velocity gradients. This means that the beads are continuously being accelerated and retarded by eddies at different velocities. Therefore, beads will frequently collide with each other either because of the differences between their own turbulent velocities, or due to the differences in their velocities relative to the (faster) moving medium surrounding them. Kusters[25] proposed expressions to estimate the collision frequency of small particles for three different eddy sizes: for eddies that are much larger than the beads; for eddies much smaller than the beads (which are those in which most of the energy is dissipated, see above) and eddies with a size comparable to that of the particles. For a more detailed discussion see Kusters[25], Spielman[39] and Hinze[18]. The effect of a collision depends on the type of collision and the energy involved in the collision ($\sim m_b v_b^2$). At collision, a compression force is induced at the point of contact. The surface is flattened and the compression force gradually decreases due to a larger contact area. The maximum is thus for the first point of contact[48]. Combining the estimation of the collision frequency according to Kusters[25] with the expression for an elastic head-on collision[48] the maximum compression stress for all collisions can be calculated by[48] $p_{max} = \Delta\rho^{2/3} \Delta v^{4/3} E^{1/3}$. The value found for the experiments described is then 150 $N/m^2$

***Rheology***

A given material deforms if a force is applied. As reaction, the material will exert a certain back force. The extent to which the material deforms depends strongly on its intrinsic properties and on how (and for how long and at which rate) the forces are applied. An elastic material deforms immediately to a certain extent and returns to its original shape after the applied stress is removed. In viscous materials the deformation remains after the stress is removed. Natural gels and many gels of synthetic polymers used for immobilization are viscoelastic materials, which means that the ratio of elastic to viscous properties depends on the time scale of the deformation. At short time scales their behaviour is mainly elastic whereas over long time scales the behaviour contains a strong viscous component. For such materials stiffness and fracture stress depend on the rate by which the material is deformed[50])

*Fracture properties*

Assuming that abrasion of gel beads occurs by continuous fracturing of the gel surface, a logical starting point for the evaluation of the factors that determine abrasion is to study the fracture properties of the material involved. Fracture is basically the result of large deformations (strains) that ultimately lead to the falling apart of the material into separate pieces. In viscoelastic polymers, fracture is accompanied by flow of (parts of) the material. This means that by applying a steadily increasing stress (force acting on a surface element divided by the area of that surface element) the material first starts to flow (locally) and fractures later. This behaviour can be studied by constructing stress-strain curves obtained by uniaxial compression at constant speed of a regular piece of gel. Typical curves of such tests are presented in Figure 1.

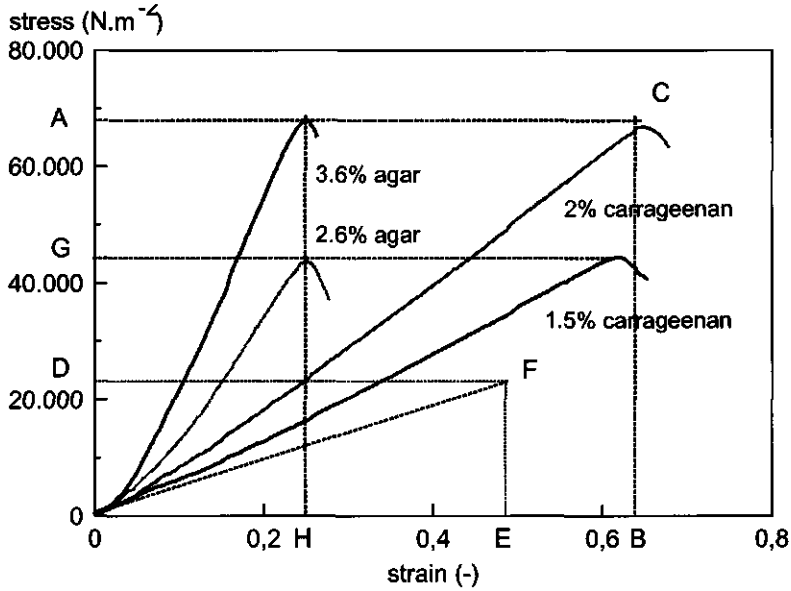


Figure 1 – Stress-strain curves of four gels (1.5% carrageenan, 2% carrageenan, 2.6 agar, and 3.6 agar) at a compression rate of 3 $mm.min^{-1}$.

As the material is being compressed (strained) it exerts an increasingly higher reaction force against the compressing agent. Beyond a certain (relative) deformation (or stress), the material can no longer resist and fractures. This point is given by the stress ($\tau_f$, A or G) and strain ($\varepsilon_f$, B or H) at fracture, respectively. The area below the curve gives the energy ($N/m^2 * m/m = Nm/m^2 = J/m^3$) needed to fracture the test piece (OCB) and includes the net energy required for fracture and the energy dissipated due to friction and flow of the

material. A measure of the ratio between the stress exerted on a material and the relative deformation of the sample (in fact a measure of the reciprocal of the deformability) is the Young's modulus represented by the ratio FE/DF in Figure 1. For most materials, this ratio depends of the deformation itself and only for (very) small deformations it is independent of it and representative for the undisturbed material.

Which properties primarily determine the mechanical stability of biocatalyst particles in a reactor is not clear yet. It is often assumed that their resistance to abrasion increases with increasing fracture stress and strain[22,24,26,35,40]., and with decreasing compression or tension modulus[32,38]. Thereby, it is implicitly assumed that if the biocatalyst particle is mechanically strong, it will be also resistant to abrasion. This has not been proved yet and it is therefore the subject of this paper. In the study of the relevance of these properties to abrasion in air-driven reactors, we also considered the fracture energy as another important parameter determining the stability of gel beads.

## MATERIALS AND METHODS

### Experimental strategy

In our hypothesis the abrasion of gel beads in a reactor occurs continuously by fracture of small pieces of the particle surface as a result of the stresses to which they are subjected. Assuming that the gels used were not too inhomogeneous[37], it can be further assumed that the local fracture properties of the gels will be of the same order of magnitude as their average values taken of the whole bead (P. Walstra, Wageningen Agricultural University, personal communication). To evaluate the importance of the stress at fracture and of the fracture energy for abrasion, combinations of gels having the same stress at fracture but different fracture energy were searched for. After selecting two combinations of gels having the desired properties at two different levels of stress at fracture, beads of these four gels were made (Fig. 1) and placed in four identical bubble-column reactors. Abrasion of the gel beads (determined by the net accumulation of sugars in the liquid bulk) was followed in time. To test the hypothesis generated by the results obtained, creep measurements in compression (to study the visco-elastic behaviour of the gels) and oscillation experiments (to evaluate the effect of cyclic compressions) were done with the selected gels and with gels of a synthetic polymer (PolyEthyleneGlycol, PEG). PEG carriers have been reported[10] to have high mechanical stability under operation in a bioreactor over a long term (five years).

**Gels used**

κ-Carrageenan (Genugel X0828) from the A/S Kobenhavns Pektinfabrik, Denmark, sodium alginate (*L.hyperborea,* Protanal LF 10/60, high-G content) from Pronova Biopolymers, Drammen, Norway and bacteriological agar No. 1 from Oxoid were purchased. PolyEthyleneGlycol (PEG) was a kind gift from Hitachi Plant Co., Japan. All gel concentrations are expressed in percentage of weight per volume (% w/v).

**Rheological tests**

For a rigorous analysis of the relation between rheological properties and abrasion, it is important to have homogeneous, reproducible gels that always exhibit the same properties under the same conditions. Therefore, reproducibility of the fracture properties was evaluated not only by measuring several samples of each material at a given deformation rate, but also by testing several batches of the gels for all deformation rates applied. Moreover, the consistency of the fracture properties with time (for instance due to swelling of the matrices or possible leaking of the gel components) was assessed by measuring these properties immediately after gel preparation and periodically afterwards during about 45 days. During this time the samples were stored in an appropriate solution. Abrasion studies were done with gel beads, but since it was much easier to measure in a reproducible way on cylindrical sample, rheological properties were determined on both types of test pieces. In that way it could also be assessed whether fracture parameters obtained with cylinders were representative for the beads tested for abrasion. Van Vliet & Peleg[50] and Luyten[27] have shown that both the sample size and shape may affect fracture properties measured.

**Preparation of test pieces**

*κ-Carrageenan* gels (at concentrations of 1,1.5, 2, 2.5, and 3%) were prepared by dispersing the appropriate amount of powder in demineralized water at room temperature using a Silverson L4R mixer. The solution obtained was heated on a hot plate until 90°C, after which KCl was added (while stirring) to a concentration of 0.134 M. Part of the gel was poured in 500 ml glass beakers and cooled at room temperature for 12 hours to allow gelation. This procedure was followed to obtain a homogeneous gel. For production of beads the remaining gel was kept at 90°C while stirring. Beads were produced by extruding the solution dropwise through a 0.6 mm diameter hollow needle (flat end) into a stirred (400 rpm) solution of cold 0.75 M KCl, in which the beads were kept for at least 60 minutes.

***Alginate*** gels (concentrations of 1,1.5, 2, 2.5 and 3%) were made following the method used by Skjåk-Bræk *et al.*[37]. Aqueous solutions of sodium alginate were prepared by dispersing the appropriate amount of alginate (Silverson L4R mixer) in a 0.2 M NaCl solution at room temperature. The gel solution obtained was poured in two dialysis membranes (length 15 cm, diameter 24 mm) and transferred to an aqueous solution of 0.05 M $CaCl_2$ (gel-inducing salt) and 0.2 M NaCl (non-gelling salt used to obtain more homogeneous alginate gels). The gels were dialyzed for 72h, refreshing the salt solution every 12h. Dialysis was used to obtain more homogeneous gels[37]. After gelation, cylindrical alginate gels with a diameter of 13 mm were obtained.

***Agar*** gels (concentrations of 1, 1.5, 2, 2.6, 3, 3.6, 4 and 5 %) were made by dispersing the appropriate amount of agar in demineralized water at room temperature followed by heating until boiling. Part of the gel was poured in glass beakers and allowed to cool to room temperature, while the remaining gel was kept at 60°C for production of beads. Beads were made by extruding dropwise the agar solution into cold demineralized water with continuous stirring. To obtain spherical beads, a 2.5 cm thick layer of viscous paraffin was placed above the water surface.

***Test pieces*** were obtained as follows. Alginate cylinders were chopped in 20 mm height cylinders. Carrageenan and agar samples were obtained by cutting the gels with a special borer, resulting in cylinders with a height and diameter of 20 ± 0.5 mm. Pieces with macroscopic structural defects were rejected. PEG cubes (20 * 20 * 20 mm) were used as received.

**Fracture properties**

***Measurements***

Force-compression curves of the test pieces were determined at 20 ± 0.5 °C in a controlled-temperature room, using a tension-compression device (Zwick table model 142510) fitted with a 2000 or 50 N load-cell for testing cylindrical test pieces and gel beads, respectively. This apparatus consists of a fixed bottom plate and a bar containing the load-cell, which can be moved at fixed speeds within the range of 0.1 - 500 $mm.min^{-1}$. The test pieces were placed on the bottom plate and compressed with a given fixed speed (0.3, 20, 30, 130 and 200 $mm.min^{-1}$). The force needed for deformation was recorded as function of time until fracturing of the cylinder or bead. A force-compression curve was obtained for each sample and stored in a file for calculation of the fracture properties. For each given gel type, gel concentration or

compression speed, 5 to 15 samples of the same batch were used. At least three different batches were used for each analysis.

***Data treatment***

Fracture stress, strain at fracture, fracture energy and Young's modulus in compression were calculated on the basis of force-compression curves obtained. *Stresses* ($N.m^{-2}$) were calculated dividing the force registered at every point by the corresponding bearing area, which is the area on which the force (moving plate) is acting. This area is changing constantly with the deformation.

For cylindrical samples and assuming that the volume of the test piece remains constant (which is reasonable within the time scale of the measurements), the actual bearing area, $A_t$, is given by: $A_t = A_0 * h_0 / h_t$ , where $A_0$ is the initial area ($m^2$), $h_t$ the height at time $t$ (m) and $h_0$ the initial sample height (m). For gel beads, the stresses were calculated considering the contact area as the area in a circle with a continuously increasing radius. This change in radius of the bearing area is given by $r^2 = r_0^2 - (r_0 - c)^2$ in which $r_0$ is the bead radius and c is the absolute value of vertical change in bead radius due to compression. For zero compression the bearing area is thus zero.

*Strain* (-) corresponds to the Hencky strain, defined as :

$$\varepsilon_H = \left| \ln\left(\frac{h_0 + \Delta h}{h_0}\right) \right| \qquad (5)$$

in which $h_0$ is the initial height and $\Delta h$ the change in height. The Hencky strain is obtained by relating any strain increase (in an already strained sample) to the changed dimension of the sample. The point in the graph at which the sample fractures (maximum of the stress-strain curve) is characterized by the (maximum) fracture stress ($\tau_f$) on the Y-axis and the accompanying fracture strain ($\varepsilon_f$) on the X-axis. The total energy required for (macroscopic) fracture is given by the area below the stress-strain curve until the fracture point. Mathematically[51]:

$$E_f = \int_{\varepsilon=0}^{\varepsilon=\varepsilon_f} \tau d\varepsilon \ (J.m^{-3}) \qquad (6)$$

The Young's compression modulus (E, $N.m^{-2}$) is the stress divided by the strain at very small deformations, here calculated from the slope of the curve at a relative strain of 5 % (between a strain of 2.5 and 7.5 %). Whenever the fracture properties of different gels, or of the same gel for different concentrations or batches, were compared with each other, Student's t-tests with 99% and 95% confidence intervals were executed.

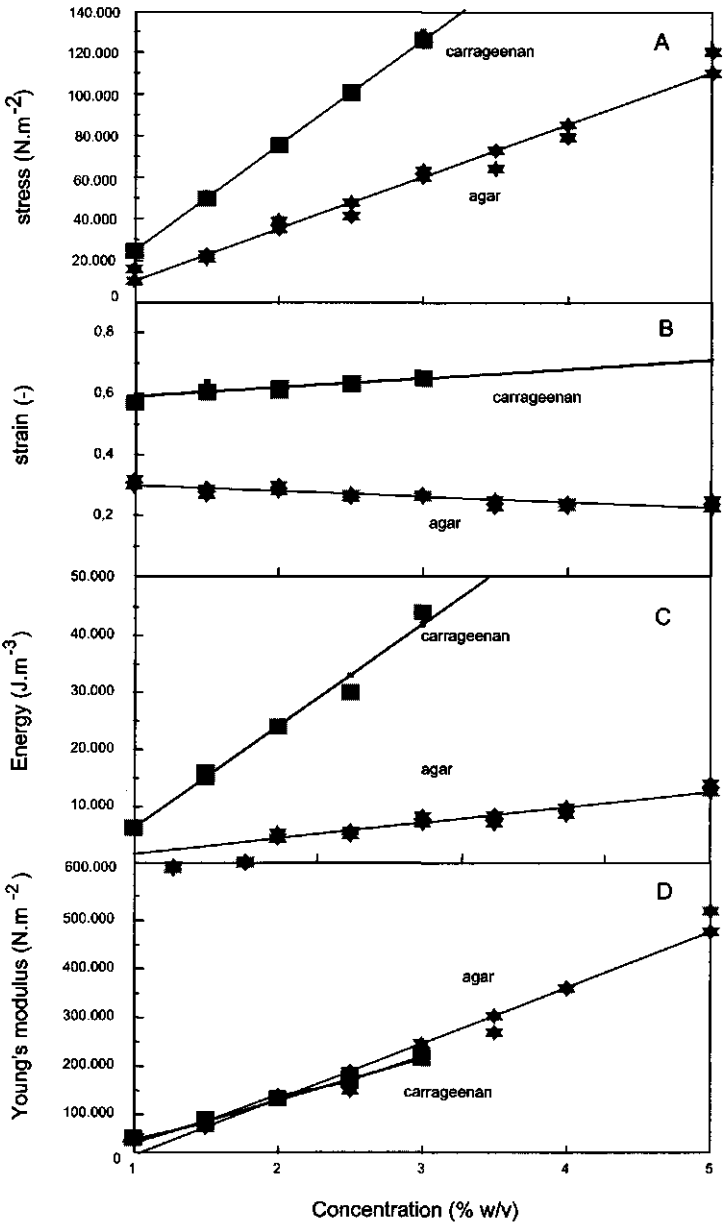


Figure 2 – Rheological properties of carrageenan and agar gels as a function of polymer concentration (% w/v). a) stress at fracture; b) strain at fracture; c) fracture energy; d) Young's modulus. Compresion speed of 3 mm/min (15% (w/v). PEG fractures at a stress of 139770 $N/m^2$ and a strain of 0.437; the fracture energy is 27154 $J/m^3$; the Young's modulus is approximately 264750 $N/m^2$).

***Creep measurements in compression***

To assess the visco-elastic behaviour of the materials used, compression and recovery experiments were done using an apparatus described by Mulder[31]. Thereto, test pieces (cylinders of 10 by 10 mm) were placed on a fixed plate and subjected to uniaxial compression due to a constant weight placed on the top of the sample. An initial pressure of 3124 $N.m^{-2}$ was applied The deformation of the test pieces was measured as a function of time by a displacement-transducer (Hewlett Packard). After 33 hours, the weight was removed and the samples were allowed to recover, which was measured by the displacement-transducer.

***Oscillation experiments***

To evaluate the effect of cyclic compressions on a gel material, oscillation tests were applied to carrageenan 1.5%, agar 2.6% and PEG cylinders. The test pieces (cylinders of 30 by 30 mm) were placed in a beaker (containing KCl at 0.134 M) that was mounted on the fixed plate of a tension-compression device (Overload Dynamics table model S100) fitted with a 2000 N load-cell. The samples were compressed at 30 $mm.min^{-1}$ until 75% of its deformation at fracture (previously determined from the stress-strain curves), after which the moving bar returned to its original position. This movement was repeated 998 times for each sample and force-compression curves were determined. The same experiment was done for a relative compression of 22.8 % of its maximum compression. Three samples were used for each determination.

**Abrasion experiments**

After selecting two combinations of each gels having the desired properties of a different fracture strain and area but similar fracture stress (see Figure 1), beads of these four gels were made and placed in four identical bubble-column reactors ($Ø_{intern}$= 99.2 ± 0.5 mm; Height = 999.4 ± 0.5 mm). Bubble columns (the most simple construction) were used instead of internal air-lift reactors to avoid possible differences in irregularities of the draught tubes, which could affect the abrasion results. Each column was filled with 6450 ml medium and 560 ml beads. The medium contained 0.134 M KCl and 2 * $10^{-4}$ % (w/v) halamid (to prevent growth of microorganisms). The beads were maintained in the columns without aeration for five days prior to the start of the abrasion experiments to allow complete swelling and release of non-bound sugars. Abrasion experiments were started by supplying humidified air at the base of the reactor through a perforated stainless-steel plate ($Ø_{pore}$ = 2 mm) at a flow of 1000 ± 10 $l.h^{-1}$. The reactors were operated for 17 days at an average temperature of 28 °C. Liquid

samples were taken twice a day for analysis of sugars, being replaced every time with an equivalent volume of fresh medium. Water lost by evaporation was replaced as well.

***Determination of the abrasion rate***

As a result of the abrasion of the gel beads (which are made of carbohydrates), polysaccharides accumulate in the reactor medium. The abrasion rate of the gel beads was thus determined by measuring the accumulation of polysaccharides in the reactor medium as a function of time. Samples of the reactor medium were taken twice a day and analyzed (in duplicate) for the presence of sugars by the phenol-sulphuric acid method described by Dubois[9] and modified as suggested by Halliwell *et al.*[14]. This spectrophotometric method is based on the development of an orange-yellow colour when polysaccharides containing (potentially) free reducing groups are treated first with phenol (80% v/v) and then with concentrated sulphuric acid. The reaction is sensitive for concentrations of the relevant polysaccharides between 5 and 50 $mg.ml^{-1}$. Accumulation of sugars in the reactor medium was corrected for the intrinsic release of carbohydrates from the beads to the liquid. For that, 10 L glass beakers containing gel beads in the same loading as in the reactors and the same medium were placed next to the columns without agitation (blanks). Samples of the beakers' media were taken and analyzed for sugars daily. Calibration curves were constructed using 5 solutions (at different concentrations) of dissolved agar and carrageenan.

## RESULTS AND DISCUSSION

The main goal of the present work was to investigate which rheological properties are relevant for the abrasion of biocatalyst particles in air-driven bioreactors. Our starting points were that the fracture properties of the support materials are important and that abrasion would occur by continuous fracturing of the bead surface. To elucidate whether fracture stress, real fracture energy or both are important, beads made of different gels having the same fracture stress and different fracture energy at two combinations (see Figure 1) were tested for abrasion in identical bubble columns under the same operating conditions.

**Selection of gels with the desired properties**

Stress-strain curves for κ-carrageenan, agar and alginate gels at concentrations varying between 1 and 5 % (w/v) were obtained at a deformation rate of 3 $mm.min^{-1}$. From each curve the stress at fracture, strain at fracture, total fracture energy and Young's modulus were calculated. Alginate gels were rejected because their fracture points were difficult

to determine. As a comparison, the fracture properties of PEG (15% w/v) were measured. At fracture, the macrocrack propagation was qualitatively very different for the three gels. PEG gels were more brittle than agar gels and these more than carrageenan. The implications of the results concerning PEG will be discussed later in this paper. The rheological properties of carrageenan and agar gels as a function of polymer concentration are shown in Figure 2. The values of PEG (15%) are given in the legends for comparison. The curves were fitted by linear regression within the range of concentrations measured.

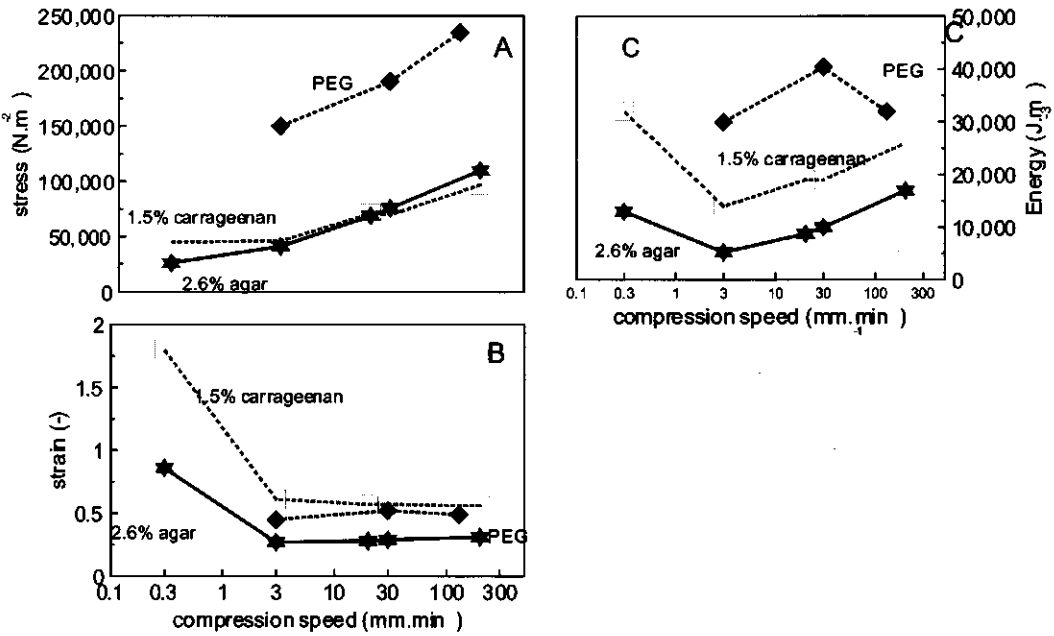


Figure 3 - Rheological properties of 1.5% carrageenan, 2.6% agar and 15% PEG gels as a function of deformation rate (compression speed). a) stress at fracture; b) strain at fracture; c) fracture energy.

The strain at fracture (Figure 2b) changed little with the polymer concentration whereas the other properties (stress and energy at fracture; Figure 2a and 2c) increased considerably. The Young's moduli (Figure 2d) were about the same for both gels and increased in a similar manner with the polymer concentration, which means that both gels react in a similar manner at very small deformations. For large deformations (e.g. stress and energy at fracture), however, their behaviour is markedly different. This illustrates that small (Young's modulus) and large (stress, strain and energy at fracture)

deformation properties need not to be related, which has already been shown by Van Vliet[51]. Therefore, conclusions about the way a material will fracture can not be based on the measurement of such small-deformation properties, nor predictions on its resistance to abrasion in a reactor can be made (see *Abrasion experiments* below).

To check the influence of the rate of applied deformation (compression speed) on the fracture properties, its effect was determined for agar (2.6%), carrageenan (1.5%) and PEG (15%) gels (see Figure 3). Generally these gels show similar patterns, in particular at deformation rates > 3mm.min$^{-1}$. Except for the energy at fracture, the dependency of PEG is quite different. We assumed therefore that (within the range tested) the fracture properties of the gels can be compared at a certain deformation rate.

Two combinations of gels having the same stress at fracture but different total fracture energy were selected based on the fracture-stress versus gel concentration curves (Figure 2a) and the fracture-energy versus gel concentration curves (Figure 2c). The stress-strain curves of these two pairs of gels (1.5% carrageenan with 2.6 % agar and 2% carrageenan with 3.6% agar) are presented in Figure 1. Their fracture properties are tabulated in Table 1. The stress at fracture of 1.5% carrageenan and 2.6% agar are comparable, while the strain at fracture is different. The same is valid for 2% carrageenan and 3.6% agar gels, while the strain at fracture for the 2 carrageenan or agar gels are comparable. These combinations are therefore suitable to determine the importance of these properties for abrasion in reactors. By choosing at least two combinations of gels, abrasion tests can be also related to different fracture energies.

**Table 1- Fracture properties of the selected carrageenan and agar gel beads.**

| | n | $\tau_f$ (N.m$^{-2}$) | $\varepsilon_f$ (-) | E (N.m$^{-2}$) | $E_f$ (J.m$^{-3}$) |
|---|---|---|---|---|---|
| 1.5% carrageenan | 9 | 44000 ± 2000 | 0.62 ± 0.02 | 63000 ± 2000 | 14000 ± 1000 |
| 2.6% agar | 5 | 43000 ± 1000 | 0.26 ± 0.02 | 140000 ± 20000 | 5000 ± 200 |
| 2% carrageenan | 6 | 65000 ± 2000 | 0.64 ± 0.03 | 90000 ± 5000 | 20000 ± 1000 |
| 3.6% agar | 5 | 69000 ±2000 | 0.24 ± 0.02 | 260000 ± 50000 | 7800 ± 200 |

The two gels of each pair were compared with each other using Student's t-tests at 99% and 95 % confidence intervals. Stress at fracture for both gels within each pair were not proven to be statistically different within both confidence intervals, whereas the fracture energies were proven to be significantly different within both intervals. Beads of these gels were made for the abrasion experiments. 1.5 and 2 % κ-carrageenan beads had a diameter of 3.30 ± 0.15 mm and 3.31 ± 0.19 mm, respectively. Agar beads 2.6 and 3.6 % were 3.86 ± 0.20 and 3.91 ± 0.17 mm in diameter, respectively. A check of the fracture properties of these beads showed that they had the same value as those of the corresponding cylindrical samples. Therefore, these gel beads were used in abrasion experiments.

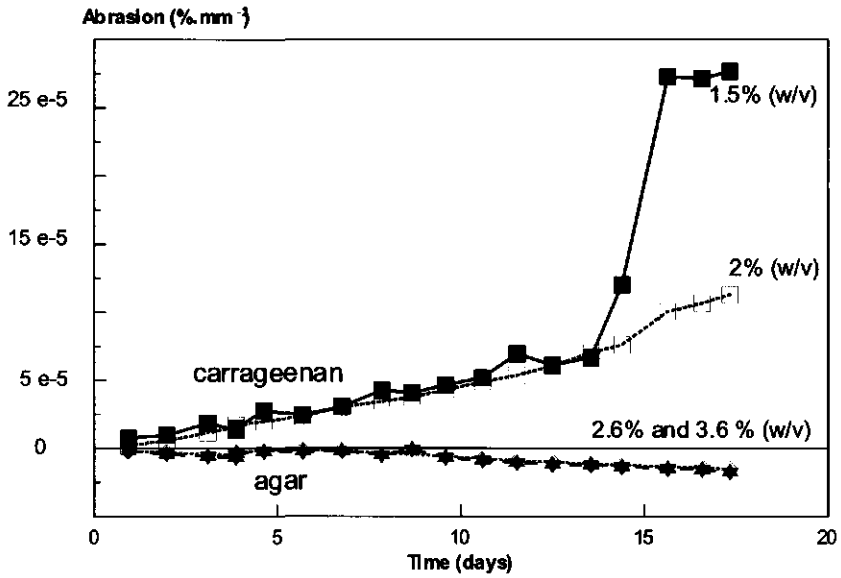


Figure 4 - Abrasion of carrageenan and agar beads in bubble columns. The % gel abraded/mm$^2$ gel bead in time.

**Abrasion experiments**

Four bubble columns containing the 4 types of gel beads were run for 17 days. For each time step, abrasion was determined by subtracting the concentration of sugars in the blanks (beakers) from those in the respective columns.

The resulting values (zero gel abrasion at time zero) were divided by the total surface of all beads in each column. Abrasion was expressed both in % $_{\text{gel abraded}}$ / mm$^2{}_{\text{gel bead}}$ and bead-diameter decrease (calculated from the total amount of gel lost) in m per day. The

total bead surface at each time step was corrected for reduction in diameter due to abrasion. The results of the abrasion experiment are shown in Figure 4. Agar gels did not abrade during the experiment.

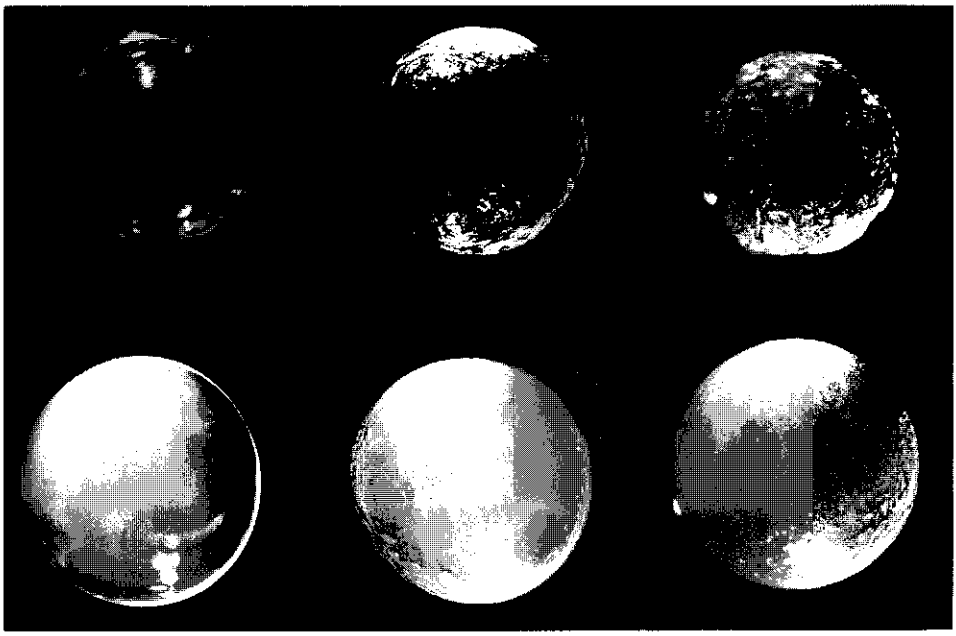

Figure 5 - The appearance of 1.5% carrageenan and 2.6% agar beads at day 0, day 10 and at the end of the abrasion experiment.

The slightly negative abrasion value from day 8 on (as a result of subtraction of the sugars released in the blanks) could not be fully explained but as this difference was very small, we considered abrasion to be zero. In contrast to agar, both carrageenan gels were liable to abrasion and that of 1.5 % carrageenan slightly more ($44.7 * 10^{-7}$ m/day, calculated over the first 13 days) than the 2% gel ($32.9 * 10^{-7}$ m/day). After day 13, the 1.5 % gel beads abraded much faster, which was also reflected in an increase in turbidity of the column medium, coalescence of air bubbles and deposition of very fine particles on the reactor walls and sparger holes. As these effects strongly affected the hydrodynamic behaviour of the bubble column containing 1.5 % carrageenan beads in relation to the other reactors (deficient sparging, larger bubbles, transition to slug-type flow) the experiment was stopped at day 18. These results were confirmed by observation of the beads. Figure 5 shows 2.6% agar and 1.5% carrageenan beads taken out of the reactor at start-up, after ten days and at the end of the experiment. At the

beginning both types of beads seemed very smooth and translucid. In the course of time, however, carrageenan beads became much rougher and less translucid than initially. At the end, large crater-like cavities were observed (as illustrated in Figure 6) and the average bead diameter decreased visibly. The surface of 2.6 % agar beads became orange-skin-like and a little less translucid as well, but much less than that of carrageenan (Figure 5). This was not expected, because the agar gels at both concentrations have a lower stress at fracture than those of carrageenan.

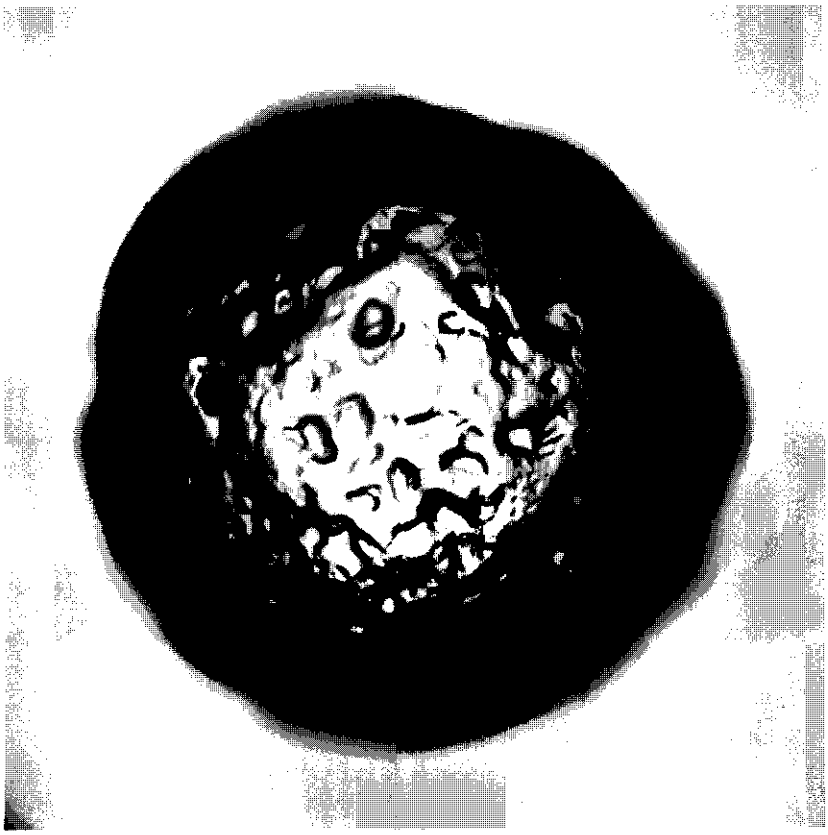

Figure 6 - A typical image of a 1.5% carrageenan bead at the end of the abrasion experiment.

From the data calculated in the Theory section, it is clear that bubble shear is not important and that none of the other possible shear mechanisms involved is clearly more important than the other; all values are in the same range. Furthermore, the magnitude of the stresses is much lower than the values needed for (macro)fracture of the beads.

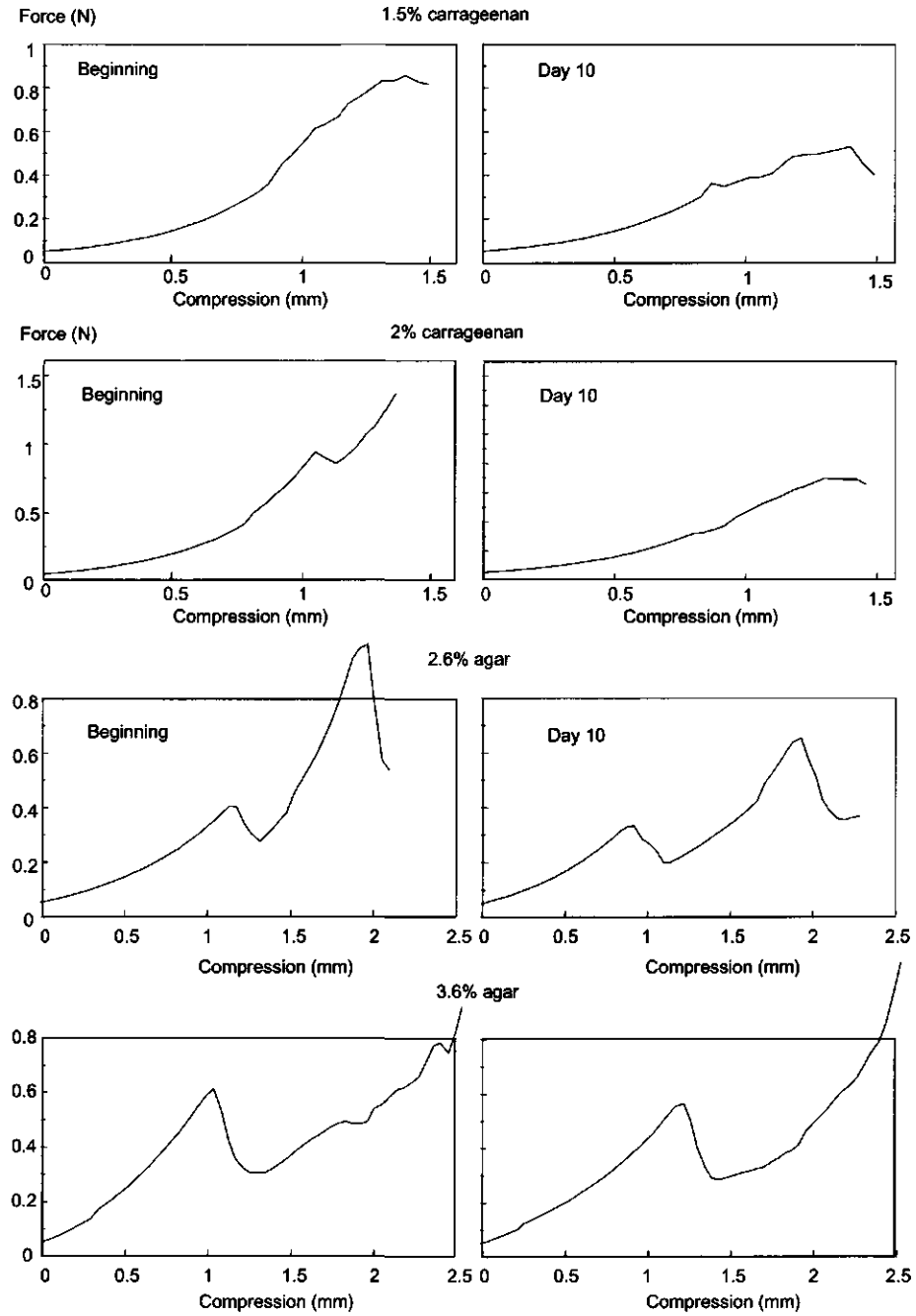


Figure 7 – Stress-strain curves for carrageenan and agar beads at day 0 and day 10 of the abrasion experiments.

Indeed, to fracture a weak gel a stress between 40000 and 60000 $N.m^{-2}$ was found while maximum shear stresses between 140-176 $N.m^{-2}$ were calculated. These shear stresses alone can therefore not explain the abrasion found in the bubble columns.
Since, at least in the beginning, the local fracture properties will be of the same order of magnitude of those for the whole bead, it seems likely that abrasion must have been caused by another effect. Possibly by repeated application of (lower) stresses to the bead surface.

To check this hypothesis, the agar and alginate gel beads were both tested for their fracture properties not only at the beginning but also after ten days. In Figure 7 the stress strain curves of carrageenan and agar beads are shown for these times. The stress at fracture of carrageenan beads clearly decreased during the experiment and that of agar seemed to decrease less, although a clear main fracture point was difficult to detect. Inspection of Figures 5 and 7 suggests that the gels are liable to fatigue or weariness in different ways, depending on the material itself and maybe on its polymer concentration. This fatigue would be caused by the repeated application of stresses on the bead surface.

**Oscillating and creep measurements**

To simulate, to a certain extent, the periodic application of stresses in a reactor (whether these are hydrodynamic stresses, collisions or both), the gel samples were subjected to repeated (1000) compressions below their fracture levels in an oscillation experiments. As comparison, polyethyleneglycol samples were subjected to the same oscillating stress. Figure 8 shows the evolution of the resistance to compression (stress) of 1.5% carrageenan, 2.6 % agar and PEG samples on applying an oscillation relative deformation equal to 75 % (9a) or 22.8 % (9b) of their macroscopic fracture strain. The resistance to compression of all three clearly decreased with the number of oscillations at both compression levels, but for carrageenan more than for agar and for this much more than for PEG. This synthetic material, though not being specially strong in terms of fracture properties (see legend Figure 2), seemed to be less liable to fatigue than agar or carrageenan. The decrease in resistance to compression decreased more by a maximum in oscillation strain of 75 % of the fracture strain than for 22.8%. Although at time-scales, magnitudes and directions other than in a reactor, the oscillating experiments somehow simulated the periodic application of stresses to biocatalyst particles in a bioreactor. As these results are expected to be related to the visco-elastic behaviour of the materials used, compression and recovery measurements were done with the three materials.

A constant weight (100g) exerting a pressure of 3124 $N.m^{-2}$ was placed on the top of the cylindrical samples of 10 mm height and deformation was registered during 33 hours after which the weight was removed. Carrageenan gels deformed more than those of agar and these much more than PEG samples (Figure 9). After releasing the weight, PEG samples rapidly reacquired their original height, whereas those of agar and carrageenan recovered only partially. Both agar and carrageenan were clearly more viscous than PEG, which was almost completely elastic over the time scale investigated. That PEG was much more elastic than the other two gels supports the idea that PEG is able to withstand better the application of the periodic applied stresses to which it is subjected in a reactor than agar and carrageenan. PEG pellets have been used in an airlift at full scale for about five years with minimal abrasion[42]. On basis of the measurement of fracture stress (a method often used to assess the mechanical strength of biocatalysts), no realistic information on the stability of the immobilized-cell supports under reactor operation would have been obtained.

**Fatigue**

The fatigue or weariness is a process that probably involves the development and propagation of microcracks during fracture. A fracture starts if somewhere in the material the local stress is higher than the adhesion or cohesion forces between the bonds of its structural elements. Thus, by fracture bonds are broken and new surfaces are formed (crack growth). Close to defects or inhomogeneities the local stresses are higher than the overall stress in the material and thus the maximum stress the material can sustain is first reached near an irregularity[12,27,52]. Since all materials have small inhomogeneities or defects, the fracture always starts at such an irregularity. When something fractures, the stress in the material around the crack can relax and the stored deformation (strain) energy can be released. Whether a crack propagates spontaneously or not depends on the balance between the differential energy released with ongoing crack growth and the differential energy required to form the new surfaces[2,27,52].
What is generally noticed as macroscopic fracture is the moment/region that the crack propegates spontaneously. Slow local growth of microcracks (irregularities), because the local stresses at a crack tip are higher than the adhesion stresses between the elements (polymer molecules) at that place (so-called fracture initiation), may start at (much) lower stresses than the in an experiment observed macroscopic fracture stress[2,27,52]. If the local stress is relieved soon it results only in a small growth of the microcrack. If such a process is repeated many times, for instance due to short lasting stresses exerted

on a bead in a bioreactor, many microcracks may develop, while a few become bigger, leading to a weakening of the gel structure. The bigger cracks may ultimately result in abrasion of pieces of gel material and the smaller ones, if their size is in the order of 0.1 μm, to a less translucid appearance[52].

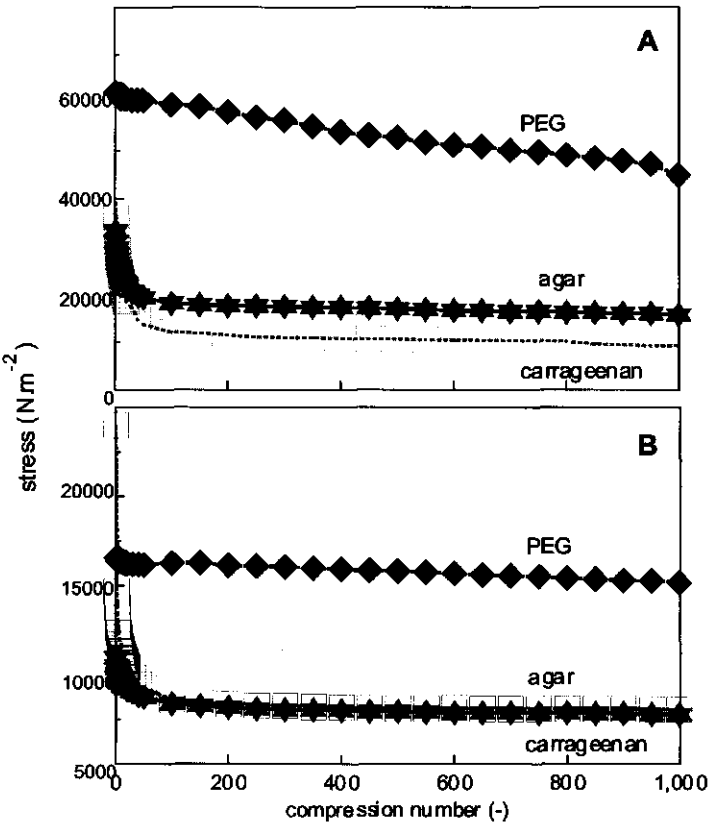


Figure 8 - The evolution of the resistance of compression (stress) of 1.5% carrageenan, 2.6% agar and 15% PEG samples at a) 75% and b) 22.8% of their maximum compression. Compression rate of 3 mm/min.

The sensitivity of a material for the formation of microcracks at stresses below the macroscopic fracture stress can generally not be deduced from the normally determined stress-strain curves in uniaxial compression until fracture. Thereto the material has to be loaded in an oscillatory way as has been done to obtain the results shown in Figure 8. To get information about the material properties determining this behaviour more fundamental studies (e.g. tension tests) on the fracture mechanics of gels in relation to

abrasion should be done. Notch-sensitivity tests in particular would provide valuable information on fracture initiation and propagation, inherent defect lengths, and on the distribution of fracture energy[51].

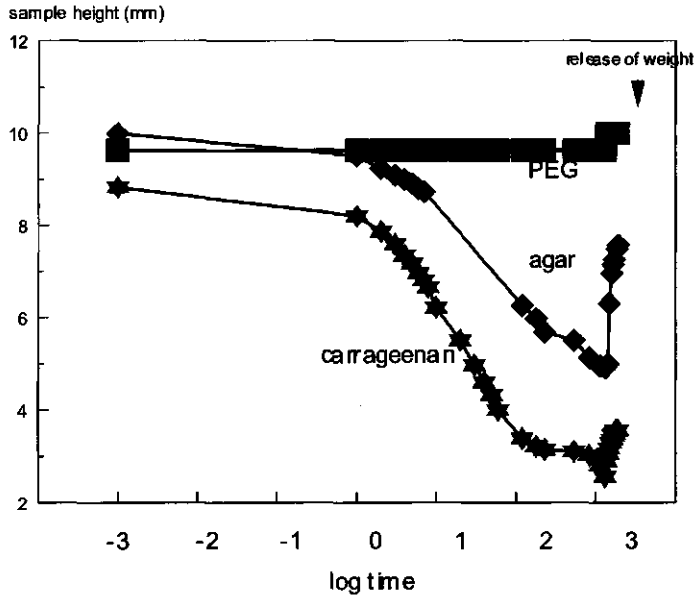


Figure 9 - Compression of carrageenan, agar and PEG gels with a constant force of 3124 $N/m^2$. After 33 hours the force was removed and the recovery determined.

The study of the resistance of gel beads to abrasion is important because beads are used for immobilization of microorganisms that carry out desired biotransformations. Growth of biomass itself, however, may also influence the resistance of the beads to abrasion. The growing colonies of cells will provide their own space within the bead at the expense of a large pressure build up. This strong internal pressure may lead to cell release by eruption and is likely to cause mechanical disruption of the surface of the gel bead[20,56]. Furthermore, the growth within the gel matrices themselves is also likely to effect their structure and mechanical properties, either by physical rupture of the structural elements due to growth itself or by gas production[15,24]. A colony of cells will form a weak spot and can strongly decrease its fracture stress[53]. Indeed, Nussinovitch *et al.*[35] have shown that both the deformability and strength of agar and alginate beads decreased considerably for high biomass concentrations.

## ACKNOWLEDGEMENTS

The authors want to thank Katja Grolle for the help with the experiments and valuable discussions and Dr. Emori (Hitachi Plant Engineering & Construction Co., Ltd., Japan) for the kind gift a PEG samples. We also aknowledge the financial support given by the Juncta Nacional de Investigação Cientifica e Tecnológica (JNICT), Portugal (BD/1505/91-F) for VAP Martins dos Santos and the financial support given by "Future treatment techniques for municipal wastewater"(RWZI-2000; 3234/3) and the Netherlands Agency for Energy and Environment (NOVEM; 51230/910) for EJTM Leenen.

## NOMENCLATURE

| | |
|---|---|
| $A_0$ | initial area ($m^2$) |
| $A_t$ | actual bearing area ($m^2$) |
| c | relative vertical change in bead radius due to compression (m) |
| $C_p$ | constant according to Baldyga[3,4] (-) |
| $d_b$ | diameter gel beads (m) |
| $d_f$ | thickness of the liquid film (m) |
| E | Youngs modulus ($N/m^s$) |
| $E_f$ | real fracture energy ($J/m^3$) |
| $F_g$ | volumetric gas flow ($m^3.s^{-1}$) |
| $h_0$ | initial sample height (m) |
| $h_t$ | sample height at time t (m) |
| $k_f$ | friction factor at the wall (-) |
| L | size of the largest, energy-rich eddies (m) (for a bubble column these are at most of the size of the column diameter, see Baldyga & Bourne[3]) |
| $m_b$ | mass of one particle (kg) |
| m | mass of reactor contents (kg) |
| n | number of samples (-) |
| <p(d)> | average pressure difference ($N/m^2$) |
| $p_{max}(d)$ | maximum pressure difference ($N/m^2$) |
| $P_s$, $P_t$ | pressure at the sparger and the top of the columns, respectively |
| r | radius of bearing area (m) |
| $r_0$ | bead radius (m) |
| R | gas constant (8.314 $J.mol^{-1}.K^{-1}$) |
| t | time (s) |
| T | Temperature (K) |
| $v_{ld}$ | mean liquid velocity on the downstream side of a resistance ($m^2.s^{-1}$) |
| $\Delta v$ | velocity difference between two colliding particles ($m^2.s^{-1}$) |
| V | molar volume of the gas ($m^3.mol^{-1}$) for a given temperature |

| | |
|---|---|
| $\langle\varepsilon\rangle$ | mean energy dissipation rate per unit mass of liquid ($m^2/s^3$) |
| $\varepsilon_f$ ; | strain at fracture (-) |
| ($\varepsilon_H$) | Hencky strain (-) |
| $\gamma$ | characteristic Kolmogoroff rate of strain (1/s) |
| $\gamma$ | strain rate ($s^{-1}$) |
| $\langle\lambda\rangle$ | size of smallest eddies (Kolmogoroff scale) when energy dissipation rate is $\langle\varepsilon\rangle$ |
| (m) | |
| $\rho$ | medium density (Kg. $^3$) |
| $\Delta\rho$ | density difference between particle and medium ($Kg.m^{-3}$) |
| $\tau$ | shear stress ($N.m^{-2}$) |
| $\langle\tau\rangle$ | shear stress when energy dissipation rate is $\langle\varepsilon\rangle$ ($N.m^{-2}$) |
| $\tau_f$ | stress at fracture ($N.m^{-2}$) |

## REFERENCES


1 Arnaud, J.P., Lacroix, C., Choplin, L. 1989. Effect of lactic acid fermentation on the rheological properties of κ-carrageenan/ locust bean gum mixed gels inoculated with *S. thermophilus*. Biotechnol. Bioeng., **34**: 1403-1408.

2 Atkins, A.G., May, J-M. 1985. Elastic and plastic fracture, Harwood Publs. Boston, USA

3 Baldyga, J., Bourne, J.R. 1995. Interpretation of turbulent mixing using fractals and multifractals. Chem. Eng. Sci. **50**: 381-400.

4 Baldyga, J, Pohorecki, R . Problems of local intermittency of turbulence in biotechnology. In: Nienow A. (ed), 3rd International conference in Bioreactor and Bioprocess Fluid Dynamics, Cambridge, UK

5 Buchholz, K. 1979. Abrasion in stirred vessels. In: Buchholz (Ed.), Characterization of immobilized biocatalysts. 136-140. Dechema Monographs, 84, Verlag Chemie, Weinheim, Germany.

6 Cherry, R.S., Papoutsakis, E.T. 1986. Hydrodynamic effects on cells in agitated tissue culture reactors. Bioprocess Engineering. **1**: 29-41.

7 Cheetham, P.S.J., Blunt, K.W., Bucke C. 1979. Physical studies on cell immobilization using calcium alginate gels. Biotechnol. Bioeng. **21**: 2155-2168.

8 Chisti, M.Y. 1989. Airlift bioreactors. Elsevier Appl.Science, New York, USA.

9 Dubois, M,, Gilles, K.S., Hamilton, J.K., Rebers, P.A., Smith, F. 1956 Colorimetric method for determination of sugars and related substances. Anal. Biochem.**28**: 350-356.

10 Emori, H., Mikawa, K., Hamaya, M., Yamaguchi, T., Tanaka, K., Takeshima, T. 1996. PEGASUS, innovative biological nitrogen removal process using entrapped nitrifiers, In: R.H. Wijffels, R.M. Buitelaar, C. Bucke, J. Tramper (eds), Immobilized cells: basics and applications. Elsevier Science, Amsterdam: 546-555.

11 Gjaltema, A., Tijhuis, L., Loosdrecht, van M.C.M. 1995. Detachment of biomass from suspended nongrowing spherical biofilms in airlift reactors. Biotechnol. Bioeng **46**: 258-269.

12 Gordon, J.F. 1968. The new science of strong materials. Penguin Books, New York.

13 Guiseley, K.B. 1989. Chemical and physical properties of algal polysaccharides used f or cell immobilization. Enzyme Microbial Technology **11**: 706-716.

14 Halliwell, G., Sakajoh, M., Dunn ,T. 1983. Microcolorimetric determination of soluble carbohydrates. Enzyme Microb. Technol. **5**: 37-40.

15 Harremoës, P., la Cour Jansen, J., Kristensen, G.H. 1980 Practical problems related to nitrogen bubble formation in fixed film reactors. Water Science Technology **12**: 253-269.

16 Havenbergh, J. van, Joos, P. 1983. The dynamic surface tension in a free falling film. J. Colloid Interface Sci. **95**: 172-182.

17 Heijnen, J.J., 't Riet, van K. 1984 Mass transfer, mixing and heat transfer phenomena in low viscosity bubble column reactors. Chem. Eng. J. **28**: B21-B42.

18 Hinze, J.O. 1971. Turbulent fluid and particle interaction. Prog. Heat. Mass Transfer **6**: 433-452.

19 Hunik ,J., Tramper, J. 1991. Abrasion of k-carrageenan gel beads in bioreactors. Proc. Internat. Symp. Environmental Biotechnology. Royal Society of Flemish Engineers, April , Oostende, Belgium.

20 Hüsken, L.E., Tramper, J., Wijffels, R.H. 1996. Growth and eruption of gel-entrapped microcolonies. In: R.H. Wijffels , R.M. Buitelaar, C. Bucke, J. Tramper (eds). Immobilized cells: basics and applications., Elsevier Science, Amsterdam: 336-340.

21 Kawase, Y., Moo-Young, M. 1990. Mathematical models for the design of bioreactors: application of Kolmogoroff's theory of isotropic turbulence. Chemical Engineering Journal **43**: B19-B41.

22 Klein, J., Eng, H. 1979. The measurement of the abrasion. In: Buchholz (Ed.), Characterization of immobilized biocatalysts. 292-299. Dechema Monographs, 84, Verlag Chemie, Weinheim, Germany.

23 Kokufuta, E., Shimohashi, M., Nakamura, I. 1988. Simultaneously occuring nitrification and denitrification under oxygen gradient by polyelectrlyte complex-coimmobilized *Nitrosomonas europaea* and *Paracoccus denitrificans* cells. Biotechn. Bioeng. **31**: 382-384.

24 Krouwel, P.G. 1982. Immobilized cells for solvent production. IBE and ethanol fermentations, Ph.D. thesis, Technical University Delft, Delft, The Netherlands.

25 Kusters, K.A, 1991. The influence of turbulence on aggregation of small particles in agitated vessels. Ph.D. thesis, Technical University of Eindhoven, The Netherlands.

26 Leenen, E.J.T.M., Martins dos Santos, V.A.P., Grolle, K.C.F., Tramper, J., Wijffels, R.H. 1996. Characteristics of and selection criteria for cell immobilization in wastewater t reatment.Water Res. **30**: 2895-2996.

27 Luyten, H. 1988. The rheological and fracture behaviour of Gouda cheese. Ph.D. thesis Wageningen Agricultural University, Wageningen, The Netherlands.

28 Martinsen ,A.., Skjak-Braek, G.., Smidsrod, O. 1989. Alginate as immobilixation material: I. correlation between chemical and physical properties of alginate gel beads. Biotechnol. Bioeng. **33**: 79-89.

29 Merchuk ,J.C.1990. Why use air-lift bioreactors? TIBTECHJ. **8**: 66-71.

30 Merchuk ,J.C., Berzin, I. 1995. Distribution of energy dissipation in air-lift reactors, Chem. Eng. Sci. **50**: 2225-2233.

31 Mulder, H. 1946. Het bepalen van reologische eigenschappen van kaas. Verslag Landbouwkundige Onderzoek, 51.

32 Muscat, A.., Beyersdorf, J., Vorlop., K-D. 1993. Poly(carbamoylsulfonate), a material for immobilization: synthesis, diffusion and mechanical properties. Biotechnol. Techn. **7**: 591-593.

33 Nienow, A.W., Conti, R. 1978. Particle abrasion at high solids concentrations in stirred vessels. Chem. Eng. Sci. **33**: 1077-1086.

34 Nilsson, I., Ohlson, S., Haggstrom, L., Molin, N., Mosbach, K. 1980. Denitrification of water using immobilized Pseudomonas denitrificans cells Eur. J. Appl. Microbiol. Biotechnol. **10**: 261-274.

35 Nussinovitch, A., Nussinovitch, M., Shapira, R., Gershon, Z. 1994. Influence of immobilization of bacteria, yeast and fungal spores on the mechanical properties of agar and alginate gels. Food Hydrocolloids, **8**: 361-372.

36 Poncelet, D., Neufeld, R.J. 1989. Shear breakage of nylon membrane microcapsules in a turbine reactor. Biotechnol. Bioeng. **33**: 95-103.

37 Skjåk-Bræk, G., Grasdalen, H., Smidsrod, O. 1989. Inhomogeneous polysaccharide ionic gels. Carbohydrate Polymers. **10**:31-54.

38 Smidsrød, O., Christensen, B.E. 1991. Molecular structure and physical behavior of seweed colloids as compared with microbial polysaccharides In: M.D. Guiry, G. Blunden (eds), Seaweed resources in Europe: uses and potential : 185-217.

39 Spielman, L.A. 1978. Hydrodynamics aspects of flocculation. In: K.J. Ives (ed.), The scientific basis of flocculation, Sijthoff and Noordhof, Alphen aan den Rijn, The Netherlands.

40 Sumino, T., Nakamura, H., Mori, N., Kawaguchi, Y. 1992. Immobilization of nitrifying bacteria by polyethylene glycol prepolymer. J. Ferment. Bioeng. **73**: 37-42.

41 Tada, M., Kimata, T., Mori, N., Emori, H. 1990. Nitrogen removal systems using immobilized microorganisms in synthetic resin. Hitachi Review **39**: 379-386.

42 Takeshima, M., Kimata, T., Mori, N., Emori, H. 1993 "Pegasus", an innovative high-rate BOD and nitrogen removal process for municipal wastewater. 66th WEF Annual Conference.

43 Tanaka, A., Nakajima H. 1990. Applications of immobilized growing cells. Adv. Biochem. Eng. **42**: 97-131.

44 Tanaka, K., Tada, M., Kimata, T., Harada, S., Fujii, Y., Mitzuguchi, T., Mori, N., Emori, H. 1991. Development of new nitrogen removal systems using nitrifying bacteria immobilized in synthetic resin pellets. Wat. Sci. Tech.. **23**: 681-690.

45 Thomas, C.R., 1990. Problems of shear in biotechnology. In: M.A. Winkler (ed.) Chemical Engineering Problems is Biotechnology, Society Chemical Industry, Elsevier Applied Science, Doordrecht, The Netherlands, pp. 23-94.

46 Thomas, D.G. 1964. Turbulent disruption of flocs in small particle size suspensions. AIChEJ. **10**: 517-523.

47 Tramper, J., Vlak ,J.M. 1988. Bioreactor design for growth of shear-sensitive mammalian and insect cells Upstream Processes: Equipment and Techniques : 199-228.

48 Bijgaart van den, H.J.C.M. 1988. Syneresis of rennet-induced milk gels as influenced by cheese-making parameters. Ph.D. thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

49 Riet, van 't K., Tramper, J. 1991. Basic bioreactor design. Marcel Dekker, New York.

50 Vliet, van T., Peleg, M. 1991. Effects of sample size and preparation, In: Rheological and fracture properties of cheese, Bulletin of the International Dairy Federation: 268.

51 Vliet, van T., Luyten, H., Walstra, P. 1992. Time-dependent behaviour of food. In: Dickinson & Walstra (Eds.) Food colloids and polymers: stability and mechanical properties, Royal Society of Chemistry, Cambridge.

52 Vliet, van T., Luyten, H., Walstra, P. 1991. Fracture and yielding of gels. In: E. Dickinson (ed.), Food polymers, gels and colloids. Royal Society of Chemists, Cambridge, UK: 392-403.

53 Vliet, van T., Walstra, P. 1995. Large deformations and fracture behaviour of gels, Faraday Discussions **101**: 359-370.

54 Wijffels, R.H., Schukking, G.C., Tramper, J. 1990. Characterization of a denitrifying bacterium immobilized in κ-carrageenan. Appl. Microbiol.Biotechnol. **34**: 399-403.

55 Wijffels, R.H., Leenen, E.J.T.M., Tramper, J. 1993. Possibilities of nitrification with immobilized cells in waste-water treatment: model or practical system? Wat. Sci. Tech.. **26**: 233-240.

56 Wijffels, R.H., Gooijer, de C.D., Schepers, A.W., Beuling, E.E., Mallée, L.F.,Tramper, J. 1995. Dynamic modelling of immobilized *Nitrosomonas europaea*: implementation of diffusion limitattion over expanding micro-colonies. Enzyme Microb. Technol. **17**: 462-471.

This chapter has been published in: Water Research, 1996, Water Research 30:2895-2996 by Leenen EJTM, Martins dos Santos VAP, Grolle K, Tramper J, Wijffels RH.

## CHAPTER 6

# Characteristics of and Selection Criteria for Support Materials for Cell Immobilization in Wastewater Treatment


## ABSTRACT

For treatment of wastewater with immobilized cells, support materials need to meet the following criteria: insoluble, not biodegradable, high mechanical stability, high diffusivity, simple immobilization procedure, high biomass retention, minimal attachment of other organisms and preferably a low cost price. In order to compare which support materials are the most suitable, characteristics of several natural and synthetic materials have been determined. For this, both literature and experimental data were used.

The immobilization procedures of natural gel materials, like alginate and carrageenan, are mild and cells grow well in these supports. Furthermore, the effective diffusion coefficients of substrates are close to those in water. These supports, however, appeared to be soluble, biodegradable and liable to abrasion. Synthetic gels, on the contrary, have better mechanical properties, but mostly lower substrate diffusion coefficients. Immobilization conditions are less mild resulting in low biomass retention. For application of entrapped nitrifying cells in wastewater-treatment systems synthetic gels, however, are promising.

## INTRODUCTION

The use of immobilized microorganisms has been gaining importance in the last few decades. In our group, nitrification and denitrification with pure strains of (de)nitrifying bacteria entrapped in gel beads have been investigated extensively to study diffusion limitation and growth of immobilized cells (Wijffels and Tramper, 1995; Wijffels *et al.*, 1991; Wijffels et al, 1995a; De Gooijer *et al.*, 1991, Hunik et al, 1994a). These studies have shown that high capacities can be reached, even under sub-optimal environmental conditions (Wijffels *et al.*, 1995b; Leenen *et al.*, 1994, Hunik *et al.*, 1994b). This has been the reason to consider the possibilities of entrapped cells not only as a model system, but as a practical system too. In a feasibility study this process with immobilized nitrifying cells was compared with activated-sludge treatment of domestic wastewater. The conclusion was: the costs for production of alginate beads are compensated by the reduced costs for construction of a smaller reactor if the support material remains stable for two months or more.

All our previous research was done with beads made of natural polymers, such as κ-carrageenan and alginate. These materials are not suitable for application in wastewater, because they are biodegradable (Østgaard *et al.*, 1993a and 1993b) and dissolve in media which do not contain their counter-ions ($K^+$ and $Ca^{2+}$, respectively; Smidsrød and Skjåk-Bræk, 1990). Furthermore, abrasion of these materials has been observed (Hunik, 1993).

The objective of this study was to select a support material that has the desired characteristics for application in domestic wastewater-treatment systems. Therefore, comparative studies with natural and synthetic support materials have been carried out, involving a large range of immobilization procedures. For this selection, both data from literature and experimental results from this study were used.

The characteristics and selection criteria of support materials for immobilization of (de)nitrifying bacteria are listed in Table I. In this study, the solubility, biodegradability, stability, diffusivity and growth in the supports were investigated in detail for alginate, carrageenan, polyvinyl alcohol, polyethylene glycol, polycarbamoyl sulphonate, and polyacrylamide.

## BACKGROUND

Immobilization of microorganisms can be defined as any technique that results in the restriction of the free migration of cells. In this study the microorganisms are entrapped in the interior of a porous gel. Two kinds of gels are studied: natural and synthetic gels.

Table I. Characteristics and selection criteria of support materials for cell immobilization

| Characteristic | Criterium |
|---|---|
| Solubility | **LOW** |
| Biodegradability | **LOW** |
| Stability | **HIGH**<br>the support should have good mechanical and rheological properties,<br>so that it is not sensitive to abrasion |
| Diffusivity | **HIGH** |
| Growth | **POSSIBLE**<br>the micro-organisms must survive the immobilization procedure and grow in the support |
| Immobilization procedure | **SIMPLE**<br>*immobilization should be possible at large scale* |
| Attachment of heterotrophic organisms | **MINIMAL** |
| Costs | **LOW** |

**Natural gels**

Alginate and κ-carrageenan, derived from algae or seaweed, are stabilized with $Ca^{2+}$ and $K^{+}$, respectively. Carrageenan consists of alternating structures of D-galactose 4-sulphate and 3,6-anhydro-D-galactose 2-sulphate. The gel strength increases with the level of 3,6-anhydro-D-galactose 2-sulphate in the polymer (Ainsworth and Blanshard, 1978). Addition of gums (Audet *et al.*, 1990; Arnaud *et al.*, 1989) or $Al^{3+}$ (Chamy *et al.*, 1990) can improve the gel characteristics. Alginate consists of D-mannuronic- and L-guluronic-acid groups and the ratio between these groups determines the gel strength. The guluronic-acid groups reacts with $Ca^{2+}$ and therefore the gel strength increases with an increasing amount of the guluronic-acid groups in the polymer, depending on the algae and the part of the species used (leave, stipe; Martinsen *et al.*, 1989; Smidsrød and Skjåk-Bræk, 1990). In general these gels allow a mild immobilization procedure, which enables most cells to survive the immobilization (Van Ginkel *et al.*, 1983; Tramper and Grootjen, 1986; Lewandowski *et al.*, 1987).

**Synthetic gels**

Several gel-forming synthetic prepolymers have been described in literature. They generally form gels by polymerization or crosslinking. This polymerization or crosslinking is, however, executed in the presence of the microorganisms to be immobilized. This environment can be rather hostile for the cells. Activity losses of more than 90% have been reported for polyacrylamide and epoxy resins (Sumino *et al.*, 1992a; Tanaka *et al.*, 1991). Milder procedures and promising techniques have been presented.

* Polyvinyl alcohol (PVA) gels are formed by crosslinking with boric acid (Hashimoto and Furukawa, 1987; Wu and Wisecarver, 1992), iterative freezing and thawing (Ariga *et al.*, 1987, Myoga *et al.*, 1991; Asano *et al.*, 1992), phosphorilization (Chen and Liu, 1994) or photocrosslinking (Ichijo et al, 1990). Most of these crosslinking reactions are still harsh, such as the boric acid method (pH 4).

* Polyurethane is a physical and mechanical stable gel, but the monomers are toxic. Activity losses of 60-90% are reported (Sumino *et al.*, 1992[a]). Vorlop *et al.* (1992) derived a less toxic poly(carbamoylsulphonate) gel (PCS) by blocking the toxic groups during the polymerization. Survival ratios of 99% were observed after immobilization (Willke *et al.*, 1994).

* Tanaka and coworkers (1991; Sumino *et al.*, 1992[b]) used a mixture of polyethylene glycol (PEG) prepolymers and a macromolecular coagulant to immobilize nitrifying sludge. The PEG pellets obtained have shown high stability, good mechanical and physical properties in a pilot-scale wastewater-treatment plant (Takeshima *et al.*, 1993).

**Stability tests**

The mechanical stability of gel beads in a reactor are largely influenced by their rheological properties, which are measured by *compression* or *tensile* tests. An example of a stress-strain curve is given in figure 1.

The mechanical characteristics deduced from these tests are:

* fracture stress (A): the ratio of the force needed to fracture the test piece over the area of the surface on which the stress was applied.

* strain at fracture (B): the relative deformation at fracture ((the difference in length between the test piece at t=0 and at fracture)/length test piece at t=0).

* rigidity modulus (CD/DE): the stress divided by the deformation at low stresses (Van Vliet, 1991[a]). Which properties are the most important is not clear yet. It is assumed, however, that the sensitivity to abrasion (gel strength) increases with the fracture stress (Sumino *et al.*, 1992[b]), the strain at fracture (deformation) and with decreasing rigidity modulus (Smidsrød and Christensen, 1991; Muscat *et al.*, 1993).

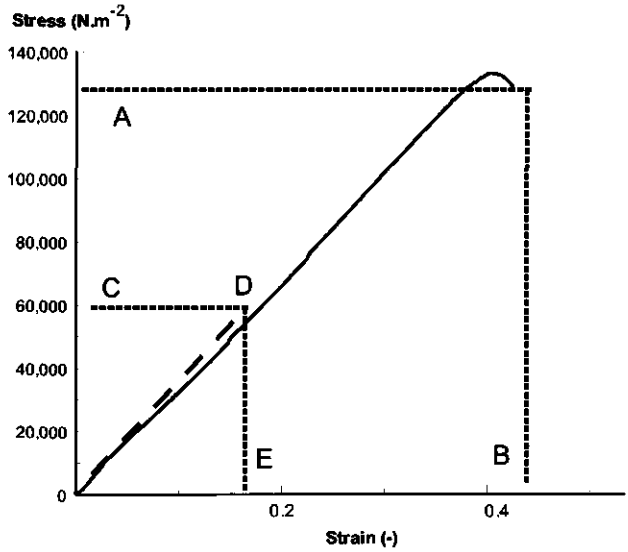


Figure 1 - Stress-strain curve of 3% κ-carrageenan with a compression speed of $3*10^{-5}$ $m.s^{-1}$.

## MATERIALS AND METHODS

### Chemicals and wastewater

Domestic wastewater from Bennekom (Department of Environmental Technology WAU, technological plant); κ-Carrageenan Genugel X-0828 from A/S Kobenhavns Pektinfabrik, Denmark; sodium alginate Manucol DM with a low guluronic-acid content (low G) from Kelco; sodium alginate HF 120 (medium guluronic-acid content; medium G) and LF 10/60 (high guluronic-acid content; high G) from Pronova Biopolymer a.s., Norway; PCS from the Federal Agricultural Research Centre, Braunschweig, Germany; PEG from Hitachi Plant Engineering and Construction Co., Japan; PVA (70000-100000 Av. Mol. Wt), locust-bean and xanthan gum from Sigma, USA. All other chemicals from Merck, Germany.

### Microorganism

*Nitrosomonas europaea* (ATCC 19718) was cultivated at 30°C in a 2.8 $dm^3$ chemostat at a dilution rate of 3.47 x $10^{-6}$ $s^{-1}$. The cultivation medium contained per $m^3$ of demineralized water: 19.0 mole $(NH_4)_2SO_4$; 0.21 mole $MgSO_4$; 5 mole $NaH_2PO_4$; 5 mole $Na_2HPO_4$; 5 mmole $CaCl_2$; 9 mmole $FeSO_4$ and 0.5 mmole $CuSO_4$. The pH was controlled to 7.4 with 7 M $NaHCO_3$. The effluent was collected in an ice bath and centrifuged at 16,300 g for 15 min at 5°C (Sorvall RC-5B Superspeed). The pellets were

washed and resuspended in 10 mM phosphate buffer (pH 7.5). This concentrated cell suspension of *Nitrosomonas europaea* was used for immobilization.

**Immobilization procedures**

Immobilization of cells was done by mixing a concentrated cell suspension of *Nitrosomonas europaea* with the polymer solution just before extrusion.

*κ-Carrageenan.* A 2.6% (w/v) polymer solution was extruded dropwise through a hollow needle by means of air pressure. The droplets were collected in a stirred KCl (0.75 M) solution and left 2 hours for hardening. The size of the droplets was adjusted by means of a longitudinal air flow (Hulst *et al.*, 1985).

*κ-Carrageenan and gums.* All experiments were executed with a 3% (w/v) total polymer concentration. Arabic, xanthan and locust-bean gum were used (8% of total polymer weight). A mixture of κ-carrageenan and the gum was extruded as described above.

*κ-Carrageenan and $Al(NO_3)_3$.* The same immobilization procedure as described above, except that the droplets were collected in a stirred $Al(NO_3)_3$ (0.15 M) solution and hardened for 15 min.

*Ca- and Ba-Ca-alginate.* A 2% (w/v) polymer solution was extruded and the droplets collected in stirred $CaCl_2$ (0.2 M) or a solution with 0.08 M $CaCl_2$ and 0.02 M $BaCl_2$ and hardened for 2 hours.

*Polycarbamoyl sulphonate.* A 10 % (w/w) PCS-solution mixed with 0.5 M $CaCl_2$ was extruded into 0.75 % (w/v) alginate (pH 8.5). Immediately a calcium-alginate layer, was formed surrounding the PCS solution. After the PCS-gelation, as result of the external pH of 8.5, the alginate layer was dissolved in a 0.1 M sodium phosphate buffer (Vorlop *et al.*, 1992).The pH of the PCS-solution was adjusted to 6.5 just before the addition of the cell suspension.

*Polyvinyl alcohol.* PVA was produced according to two methods:

- *Iterative freezing and thawing.* An 8 or 10% PVA-solution was extruded into liquid nitrogen. Beads were instantaneously formed and frozen. After this, thawing (4°C) and freezing (-30°C) was repeated 1 or 2 times.
- *Boric-acid method.* A 10% PVA-solution was extruded into a stirred saturated boric-acid solution (pH 4) and the drops were hardened for 24 hours. This was also done by extruding into a saturated borax ($Na_2B_4O_7.10H_2O$) solution.

*Polyethylene glycol.* Cylindrical pellets were a gift of Hitachi Plant Engineering and Construction Co., Japan.

**Solubility**

The solubility of alginate gels was investigated in several solutions: demineralized water; solutions with different concentrations of $CaCl_2$ and NaCl or Ca- and Na-phosphate; and domestic wastewater.

Hundred gel beads were shaken in 30 ml solution in an erlenmeyer flask at 100 rpm and the solution was refreshed daily. Periodically the bead diameter and the force to fracture beads (see below) were measured.

The experiments in wastewater were also done in the presence of 0.0025 mM $NaN_3$ (toxic to organisms), to determine if the beads dissolved or were biodegraded.

**Stability**

Rheological properties were measured by *compression* and *tensile* tests of at least 8 test pieces. Tensile tests were done, because sometimes no fracture could be accomplished by compression.

*Compression* was done with a 2000 N load cell with a constant speed ($3 * 10^{-5}$ $m.s^{-1}$) at room temperature. The resistance of the material was registered until the beads fractured, and the force necessary to fracture the test piece, the fracture stress, strain at fracture and the rigidity modulus were determined (Van Vliet, 1991b).

*Test piece preparation.* Cylindrical and cubic test pieces, as well as beads were used. The carrageenan test pieces were prepared by heating the polymer solution to 90°C, mixing with KCl in a glass beaker and cooling to room temperature. After gelation the gels were cut in cylinders of 20x20 mm. The alginate test pieces were made in a dialysis membrane put in the $CaCl_2$ solution. After gelation the gels were cut into cubes of 10x10x10 mm. The PVA test pieces were prepared in a plastic beaker by freezing in liquid $N_2$ for 1 hour and storing for 12 hours at -18°C. Thawing was done at 4°C. Repetitive freezing and thawing was done at -18°C and 4°C. The PCS test pieces were prepared by increasing the pH of a 10% PCS-solution until gelation occurred. The PVA and PCS test pieces were cut in cylinders of 20x20 mm and the PEG test pieces were cubes of 10x10x10 mm.

*Tensile* tests were done with an elongation apparatus (Muscat *et al.*, 1993). The test piece was pulled with a constant speed ($2.1 * 10^{-5}$ $m.s^{-1}$) at room temperature and the resistance of the material registered. The rigidity modulus and the strain at fracture were determined.

*Test piece preparation.* The test pieces (gel strips) were cut from the gel prepared in a petri-dish or a Teflon plate (thickness $3*10^{-3}$ m).

**Growth**

Immobilized *N. europaea* cells (2.0 g beads) were incubated in 50 ml cultivation medium at 30°C and shaken at 80 rpm. The maximal oxygen consumption was measured periodically according to Van Ginkel *et al.* (1983).

**RESULTS AND DISCUSSION**

In this study several materials for entrapment of cells were compared. This was done according to the criteria described in Table I.

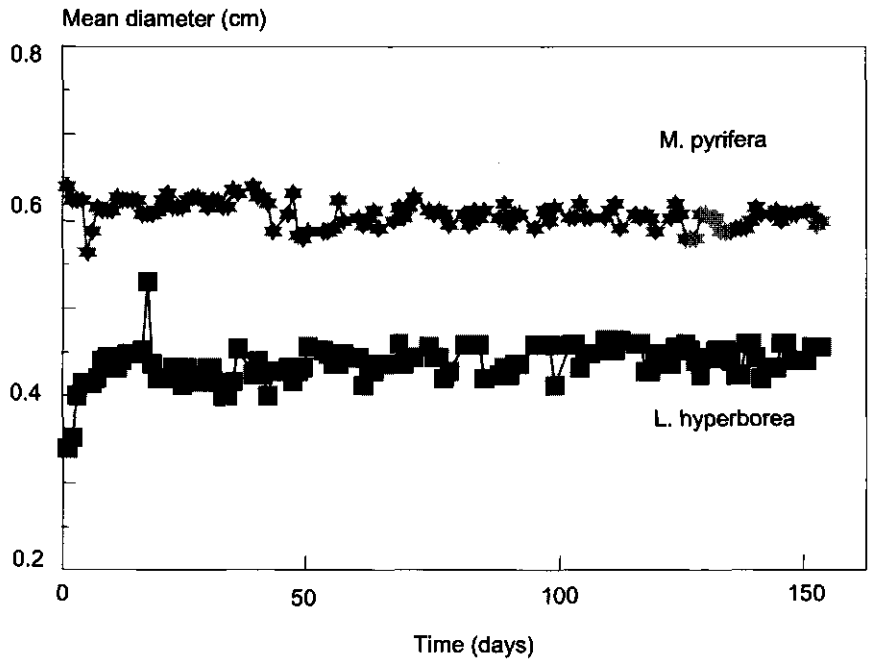


Figure 2 - Solubility of Ba-Ca-alginate gel beads of *M. perifera* and *L. hyperborea* in demineralized water. Mean diameter of gel beads as a function of time.

**Solubility**

*Natural gels*

Ca-alginate dissolves within a few days in domestic wastewater (results not shown). By adding $Ba^{2+}$ as counterion a more stable gel can be formed. Further optimization of alginate gels was done by using alginates with different amounts of guluronic acid. The solubility of Ba-Ca-alginate with a high (*Laminaria hyperborea*) and a low guluronic acid content (*Macrocystis pyrefera*) was tested in demineralized water (Figure 2). Both gels did not dissolve during 160 days.

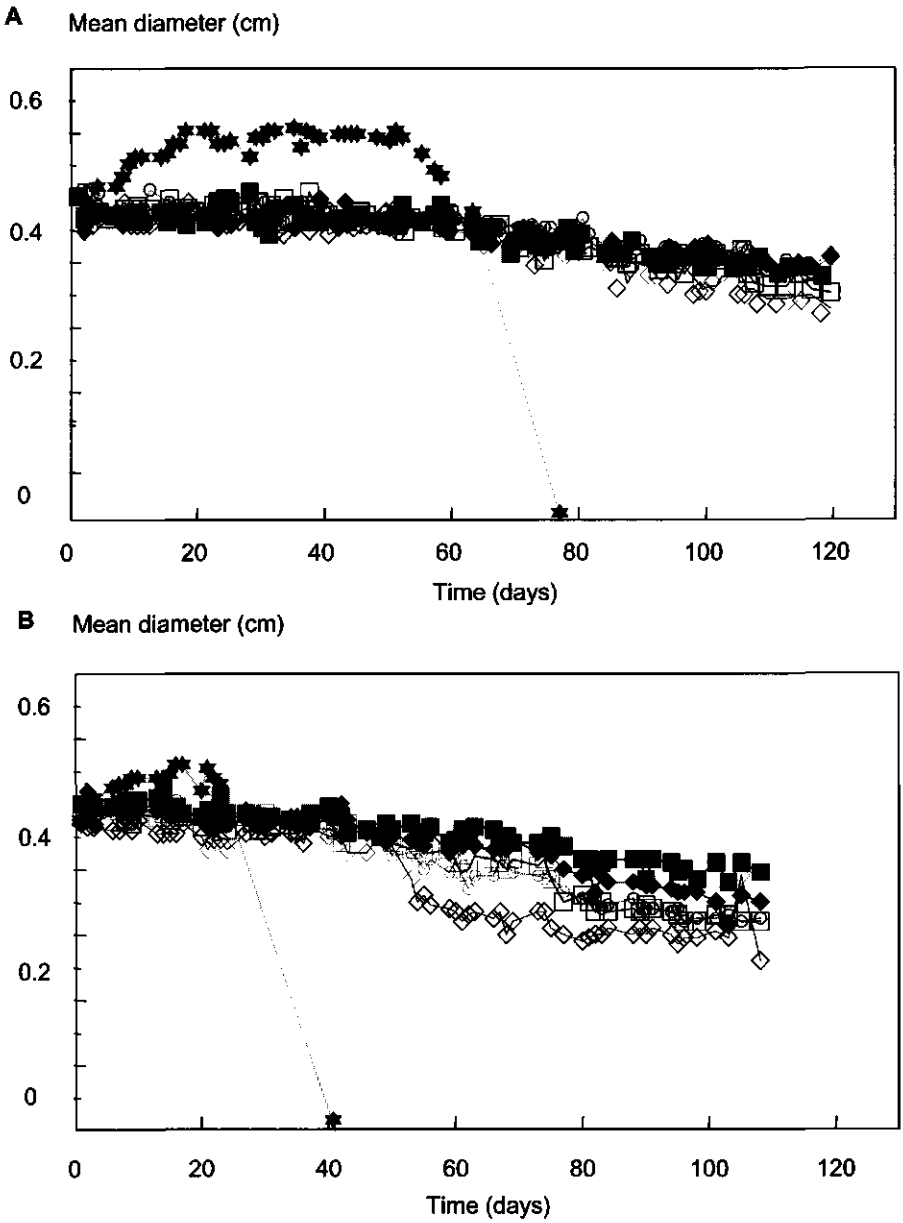


Figure 3 - Solubility of Ba-Ca-alginate of *L. hyperborea* in A) solution with different $NaCl/CaCl_2$ ratios. B) solutions with different Na/Ca-phosphate ratios. Mean diameter of gel beads as a function of time. (✶10 mM $Na^+$; ■ 8 mM $Na^+$, 2 mM $Ca^{2+}$; □ 6 mM $Na^+$, 4 mM $Ca^{2+}$; ○ 4 mM $Na^+$, 6 mM $Ca^{2+}$; ▼ 2 mM $Na^+$, 8 mM $Ca^{2+}$; ◇ 10 mM $Ca^{2+}$).

The solubility of alginate gels depends on the $Na^+/Ca^{2+}$ ratio in the medium. The counterions in the gel can exchange with the $Na^+$ in the medium and the gel will dissolve. Therefore, the optimal Ba-Ca-alginate was incubated in solutions with different $NaCl/CaCl_2$ ratios (Figure 3A). This gel slowly dissolves if 2 mM $Ca^{2+}$ was present (≈ 20% in 120 days), independent of the $Na^+$ concentration. If only NaCl was present the gel dissolved completely within 70 days.

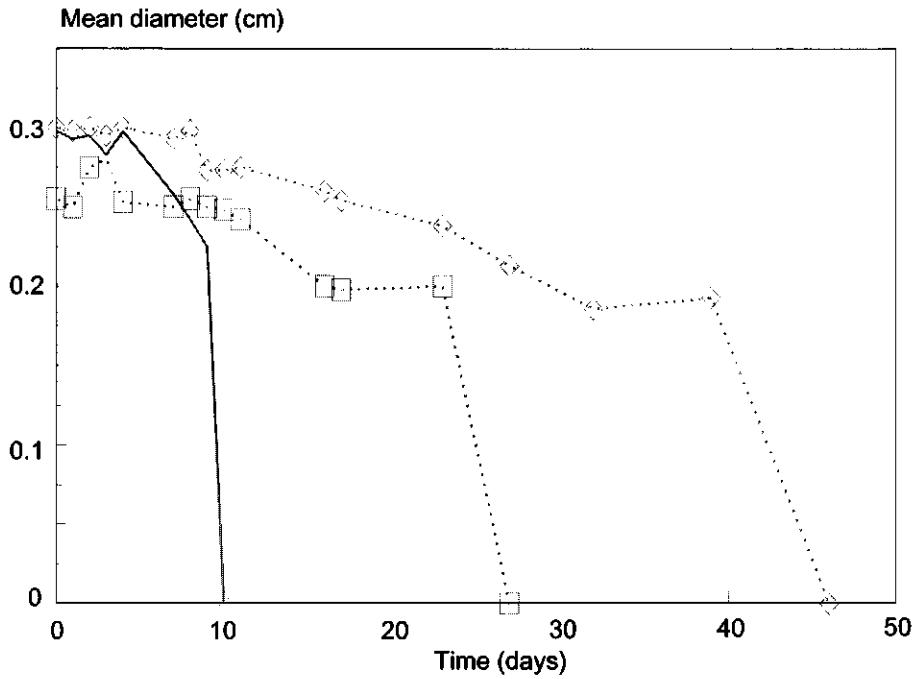


Figure 4 - Solubility of Ba-Ca-alginate gels in domestic wastewater. Mean diameter of gel beads as a function of time (O low; □ medium; — high G content).

Chelating compounds like phosphates or citrates can extract the $Ca^{2+}$ from the matrix and enhance solubility. Figure 3B shows the experiment done with different Ca/Na-phosphate ratios. Approximately the same results as Figure 3A were found, except that the beads dissolved faster. Martinsen *et al.* (1989) reported that this alginate gel was stable at $Na^+/Ca^{2+}$ ratios of 30/1, while other types of alginate gels already dissolve at a ratio of 5/1. Figure 2 showed that alginate gels did not dissolve in absence of $Ca^{2+}$. However, as any chelating or destabilizing compound is present $Ca^{2+}$ need to be added. In domestic wastewater there are many components. The molar ratio of $Na^+/Ca^{2+}$ varies between 5/1 and 9/1 in the Netherlands. It is therefore not expected that this Ba-Ca-alginate gel dissolves in domestic wastewater. The solubility of the optimal Ba-Ca-alginates in

domestic wastewater was investigated (Figure 4). In contrast to what was expected, all gels dissolved during the experiment. Probably, the amount of chelating compounds, like phosphates or citrates, was so high that the gels dissolved. The solubility of Ba-Ca-alginate gel beads decreased with an increasing amount of guluronic acid. The lifetime ranges from 3 to 40 days.

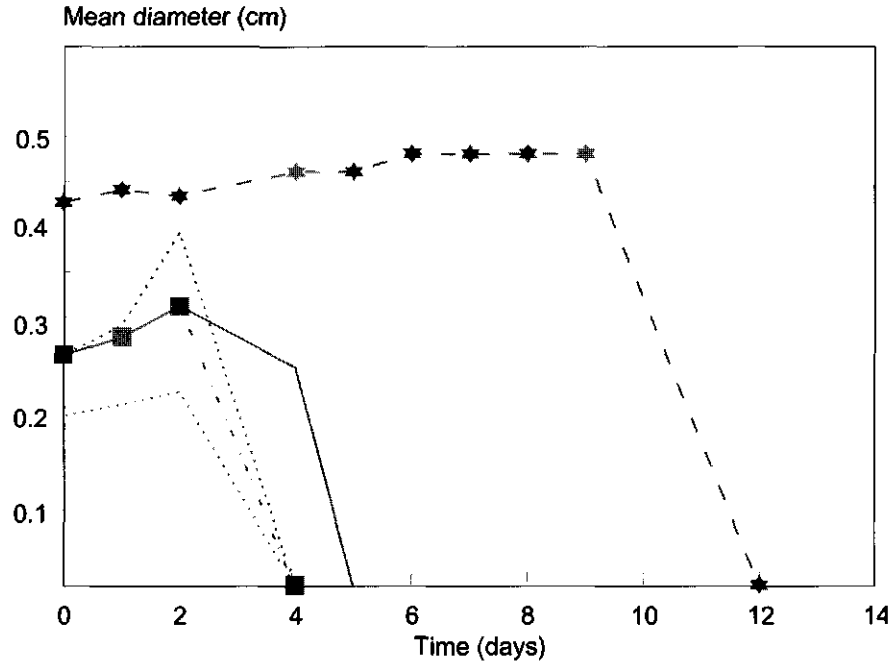


Figure 5 - Solubility of κ-carrageenan-based gels in domestic wastewater. Mean diameter of gel beads as a function of time (O pure; □ + $Al(NO_3)_3$; _ + arabic gum; ● + xanthan gum; ■ + locust bean gum).

Also κ-carrageenan beads were incubated in domestic wastewater. They dissolved within 3 to 4 days (Figure 5) due to the lack of stabilizing counterions ($Ca^{2+}$ or $K^+$). Solubility of carrageenan gel beads can be decreased by addition of a gum interacting with the carrageenan polymer (Audet *et al.*, 1990; Arnaud *et al.*, 1989) or $Al^{3+}$ (Chamy *et al.*, 1990). In Figure 5 the solubility of the various carrageenan-based gels in domestic wastewater is shown. All gels dissolved within a few days. Addition of xanthan, however, tripled the life-time of the gel beads under these circumstances. To check whether the gel beads dissolved or were biodegraded by organisms present, the same experiments were repeated in the presence of $NaN_3$. The same results were obtained; thus biodegradation of the gels can be excluded.

*Synthetic gels*

In Figure 6 the solubility of PCS, PVA (frozen 3 times) and polyacrylamide gel beads is shown. PCS and PVA did not dissolve in domestic wastewater, while polyacrylamide dissolved slowly (≈ 12% in 70 days). To investigate if the characteristics of the gels altered during the experiment the fracture force was determined periodically too. If this changes the gel composition is changed too or the gel is weakened due to abrasion. The fracture force did not change (shown for PVA in Figure 7). The gels were physically stable during the exposure time. PEG was not tested by ourselves. In Japan this material is already used in practice for several years for the purification of domestic wastewater (Takeshima *et al.*, 1993), thus solubility in domestic wastewater can be excluded. Natural support materials dissolved in domestic wastewater, while synthetic materials were physically stable during the exposure time.

**Biodegradability**

In wastewater-treatment systems an abundant population of organisms is present. If any of these organisms can degrade the support material the immobilized-cell process can not be applied. In the experiments described above no degradation of support material was found, but this does not mean that these gels are not biodegradable at all. In marine environments several organisms (molluscs, bacteria, fungi) that contain carrageenan- or alginate-degrading enzymes were found (Østgaard 1993a; 1993b).

Biodegradation of the synthetic gels has not been reported in literature. As said, no biodegradation of PEG was found (Takeshima *et al.*, 1993). Polyurethane is often used in domestic wastewater-treatment systems without biodegradation of the material (Stormo and Crawford, 1992; Sumino et al, 1992[a]). As PCS is a derivative of polyurethane biodegradation of PCS is not expected. Asano *et al.* (1992) and Myoga *et al.* (1991) used PVA beads hardened by iterative freezing and thawing in wastewater-treatment systems and never reported biodegradation. Natural support materials may be biodegraded but, so far, no biodegradation of synthetic support materials has been reported.

**Stability**

In this study, it is assumed that the gel strength increases with the fracture stress, strain at fracture and with decreasing rigidity modulus. These rheological properties were determined with compression and tensile tests (Figure 8). The fracture stress and rigidity modulus increase with increasing amount of polymer in the gel and does not influence the strain at fracture (results of carrageenan). It is not possible to conclude that the gel strength increases too.

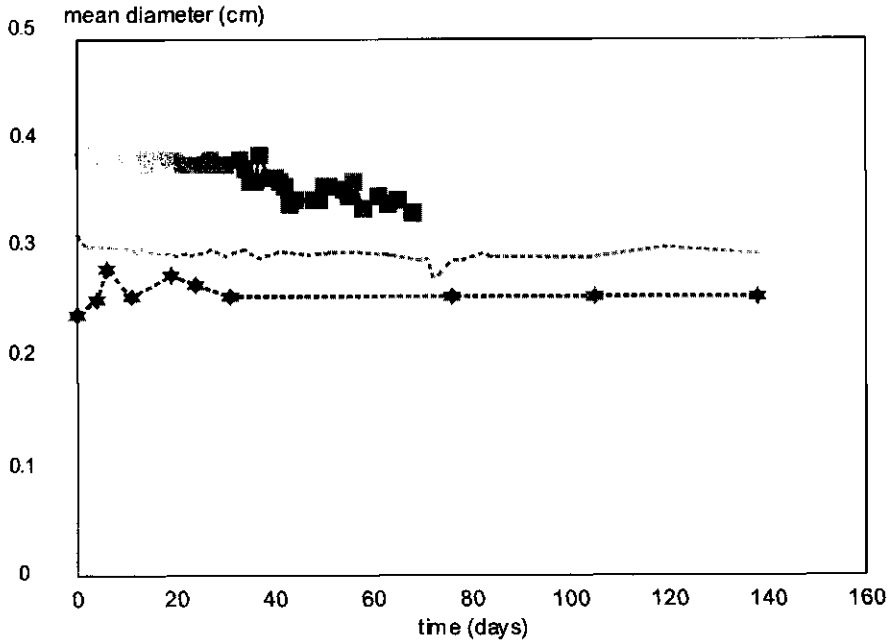


Figure 6 - Solubility of synthetic gels in domestic wastewater. Mean diameter of gel beads as a function of time (O PVA; ● PCS; ■ Polyacrylamide).

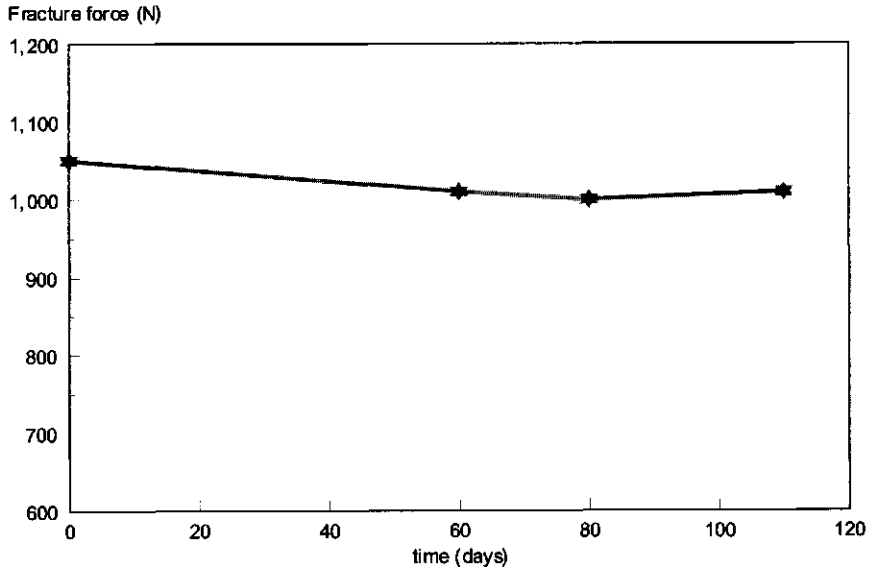


Figure 7 - The fracture force of PVA gel beads (10% w/v, twice frozen and thawed) during exposure to domestic wastewater

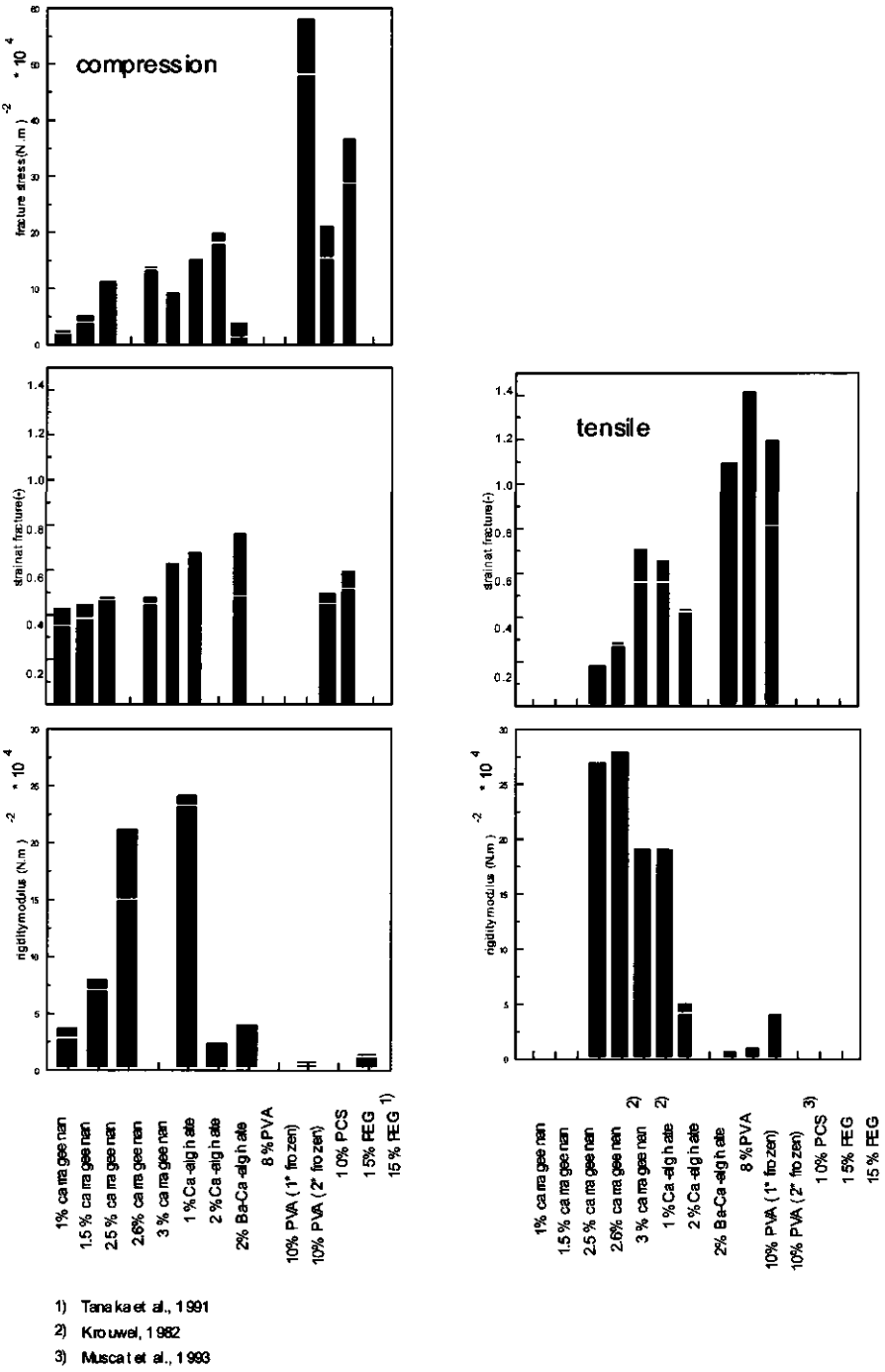


Figure 8 - Fracture stresses, strains at fracture, and rigidity moduli of support materials. A) Compression tests; B) Tensile tests (■ standard error bar).

Hunik and Tramper (1993) reported that carrageenan beads abraded. As all natural gels tested have values in the same range, it is expected that they all are sensitive to abrasion. The high-G Ba-Ca-alginate, however, has a somewhat higher value for the fracture stress and the rigidity modulus is much lower, and thus is expected to be less sensitive to abrasion.

The synthetic supports have higher fracture stresses, higher strains at fracture, and lower rigidity moduli than the natural supports. Only a few test pieces of PVA (frozen once) could be fractured and therefore the fracture stress and strain could not be determined accurately. The value of the fracture stress for PEG is a bit higher than the value for the high-G Ba-Ca-alginate. PEG is already used in a large-scale wastewater-treatment plant in Japan and more than 5 years of physical gel durability has been demonstrated (Takeshima *et al.*, 1993). Therefore, other rheological properties might be important.

In general it can be concluded that the synthetic gels have preferred rheological properties and are probably more resistant against abrasion. Presently, we are studying the relation between rheological properties of a support material and the abrasion of such material.

**Diffusivity**

The effective diffusion coefficient of glucose and oxygen were chosen to compare the diffusion of substrates in matrices (Table II). Only literature data were used. The values for the natural gels are comparable; the diffusion coefficient of oxygen is between 1.6 and $2.0*10^{-9}$ $m^2.s^{-1}$ and of glucose around $0.6*10^{-9}$ $m^2.s^{-1}$. For comparison, the diffusion coefficient of oxygen and glucose in water are $2.83*10^{-9}$ $m^2.s^{-1}$ (Wise and Houghton, 1966) and $0.673*10^{-9}$ $m^2.s^{-1}$ (Weast, 1979), respectively.

The values for the synthetic gels differ. Generally a high polymer concentration, which can hamper the substrate transport, is needed to form a strong gel. The transport of oxygen in photocrosslinked PVA (PVA-SbQ) or polyurethane gel is much lower, the transport of glucose into PVA (iterative freezing/thawing) is comparable with that in natural gels; the value of PCS is somewhat lower. In general the diffusivity is higher in natural gels than in the described synthetic gels. So more substrate can be transferred to cells growing in the interior of the support.

**Table II. Effective diffusion coefficients of oxygen and glucose in gel matrices.**

| gel | % polymer | $D_{O2}$ $*10^{-9}$ $m^2.s^{-1}$ | $D_{glucose}$ $*10^{-9}$ $m^2.s^{-1}$ | reference |
|---|---|---|---|---|
| Ca-alginate (lowG) | 1 | 1.75 | | Hulst, 1989 |
| | 2 | 1.98 | | Hulst, 1989 |
| | 3 | 1.79 | | Hulst, 1989 |
| Ca-alginate | 2 | 2.11 | | Adlercreutz, 1986 |
| | 2 | | 0.66 | Tanaka *et al.*, 1984 |
| | 2 | 2.3 | | Kurosawa et al, 1989 |
| κ-carrageenan | 1 | 1.58 | | Hulst, 1989 |
| | 3 | 1.58 | | Hulst, 1989 |
| | 3 | 2.05 | | Wijffels *et al.*, 1995[b] |
| | 5 | 1.87 | | Hulst, 1989 |
| PVA 2* freeze-thaw | 10 | | 0.66 | Ariga *et al.*, 1994 |
| PVA-SbQ | | 1.0-0.7 | | Renneberg *et al.*, 1988 |
| PVA-alginate | 8/2 | 1.5 | | Shindo and Kamikura, 1990 |
| PCS | 10 | | 0.5 | Muscat *et al.*, 1993 |
| Polyurethane-3 or 6 | | 0.19 | | Renneberg *et al.*, 1988 |

**Growth of nitrifying bacteria in gel matrices**

Cells have to survive the immobilization procedure and grow in the formed matrix. Growth of nitrifying cells entrapped in natural gels has been reported extensively (Van Ginkel *et al.*, 1983; Hunik *et al.*, 1994a; Tramper and Grootjen, 1986; Wijffels *et al.*, 1991).

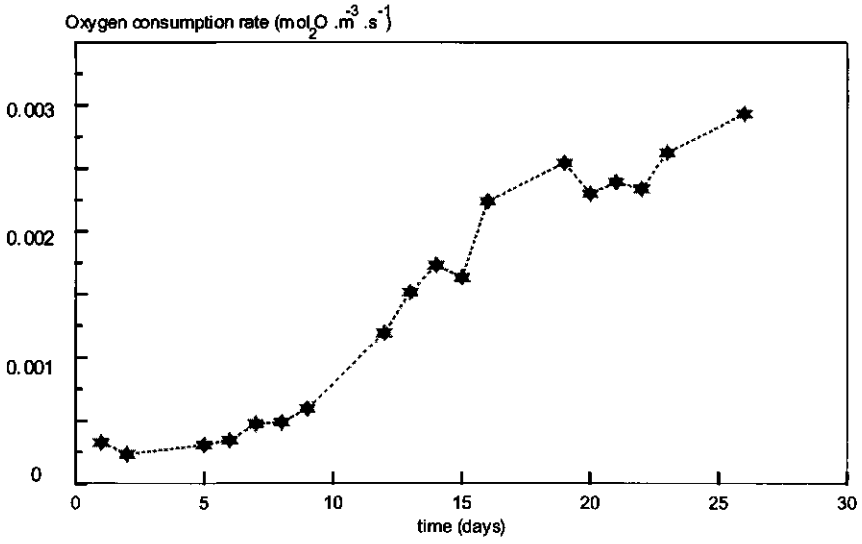


Figure 9 - Growth of *Nitrosomonas europaea* in PCS. The maximal oxygen consumption rate versus time.

The procedures to prepare synthetic gels are generally more hostile and often cause cell death. Growth of *Nitrosomonas europaea* in PCS was observed (Figure 9), but the start-up phase was long. During polymerization the pH dropped rapidly, probably resulting in cell death. In Figure 9 can be seen that after some time the maximal oxygen consumption rate (a measure for the overall viable biomass) increased. Willke *et al.* (1994) also reported successful immobilization in PCS.

The PVA/boric-acid method was tested, but due to the low pH no cells survived the procedure. The toxic effect of boric acid was reduced by using borax instead. This resulted in a gel, that dissolved within a few hours. Activity of activated sludge (Hashimoto and Furukawa, 1987) and of *Pseudomonas* sp. (Wu and Wisecarver, 1992) after immobilization with the boric-acid method were however reported. Chen and Lin (1994) established growth of denitrifying sludge in phosphorylated PVA gel, obtained by crosslinking with boric acid followed by esterification with phosphate.
Alternatively, the iterative freezing and thawing procedure was tested as well. Slow growth of *Nitrosomonas europaea* was found after freezing and thawing once. Probably by optimizing this procedure, for example by using a cryoprotectant, growth of cells may be successful. Presently, immobilization of *Nitrosomonas europaea* in PVA by iterative freezing and thawing is studied further. Growth of a mixed culture of nitrifying bacteria

Willke and Vorlop (1995), *E. coli* (Ariga *et al.*, 1987) and acclimated sludge (Ariga *et al.*, 1987; Myoga *et al.*, 1991) immobilized by the PVA-freezing method have been reported. PEG pellets showed good nitrification capacities in domestic wastewater (Tanaka *et al.*, 1991; Takeshima *et al.*, 1993).

In general growth of *Nitrosomonas europaea* is easier to obtain in natural gels than in synthetic support materials. Optimization of the immobilization procedures for the synthetic gels may result in useful support materials for application in wastewater-treatment systems.

**Immobilization procedure**

For application of immobilized-cell systems, the immobilization procedure should be easy and upscaling possible. Klein and Vorlop (1983) were able to scale-up the extrusion technique by substituting one outlet with 42. Hulst *et al.* (1985) developed a technique, which consists of breaking up a jet of the biocatalyst/natural-polymer mixture in uniform droplets with a resonance-nozzle. Gotoh *et al.* (1993) improved this method for suspensions with high viscosities.

**Table III. Characteristics of gel materials for immobilization.**

| characteristic | Natural gels | | | Synthetic gels | | |
|---|---|---|---|---|---|---|
| | carrageenan | Ca-alginate | Ba-Ca-alginate | PVA | PCS | PEG |
| solubility | high | high | high | low/not | low/not | low/not |
| biodegradability | possible | possible | possible | low | low | low |
| stability | low | low | medium | high | high | medium |
| diffusivity | ++ | ++ | ++ | + | +/- | ? |
| growth | + | + | + | +/- | +/- | + |
| immobilization procedure | simple | simple | simple | laborious | laborious | simple |

\- = bad; not stable
+/- = moderate
\+ = good
++ = very good
? = not determined or reported

Further scale-up of the extrusion technique was done by Hunik *et al.* (1993). Ogbonna *et al.* (1989) used a rotating-disc atomizer, that disperses an aqueous alginate solution in air. Poncelet *et al.* (1992) emulsified an alginate suspension in vegetable oil. The diameter of the beads was controlled by the impeller and rotational speed. The described techniques are especially useful for the natural gels.

For synthetic gels, prepared by an extrusion technique, these techniques can in principle be used too. In case of immobilization in PVA by iterative freezing, the other steps involved still have to be optimized. Immobilization in PCS is laborious: during the polymerization the mixture becomes more viscous, which hampers the ease of handling drastically. More research is necessary before mass production of PCS beads can be done successfully. Two relatively simple procedures are used for immobilization in PEG: 1) PEG is mixed with alginate and extruded through a hollow needle in a stirred $CaCl_2$-solution; after alginate-PEG beads are obtained the alginate is dissolved, resulting in PEG beads. 2) Large quantities of PEG pellets are made by pouring a sheet of PEG and cutting this sheet into small cubes (Takeshima *et al.*, 1993).

## CONCLUSIONS

The characteristics of the investigated gel materials are summarized in Table III. Natural gels may be suitable material for some applications: cells survive the immobilization procedure and can grow in the support, the diffusion coefficients are high, and the immobilization procedures easy to scale-up. They are, however, soluble, biodegradable and relatively weak and therefore not suitable for treatment of domestic wastewater. Synthetic supports, on the other hand, do not dissolve, are not biodegradable and have good mechanical properties. The diffusivity is lower and the immobilization procedures more harsh and difficult than for the natural supports. The survival of cells in these supports is therefore poor. It is possible to obtain growing cells in these supports, but optimization is necessary.

Summing up, the synthetic gels (PVA, PCS and PEG), investigated in this study, are the more promising materials for application in wastewater-treatment systems.

## ACKNOWLEDGEMENTS

This work was funded by "Future treatment techniques for municipal wastewater" (RWZI-2000) and the Netherlands Agency for Energy and Environment (NOVEM). The authors thank Ana-Teresa da Silva-Ferreira, Teresa Fernandes, Zhang Li-Ru, Catrinus van der Sluis, Mireia Ripoll-Martinez and Ida Günther for their help with the experiments, Dr. Ton van Vliet for the fruitful discussions, Prof. Klaus Vorlop and Andreas Muscat (FAL

Braunschweig, Germany) for the supply of PCS, and Hirohoshi Emori (Hitachi Plant engineering and Construction Co., Japan) for the supply of PEG.

## REFERENCES

Adlercreutz P (1986) Oxygen supply to immobilized cells: 5. Theoretical calculations and experimental data for the oxidation of glycerol by immobilized *Gluconobacter oxydans* cells with oxygen or p-benzoquinone as electron acceptor, *Biotech. Bioeng.* 28: 223-232.

Ainsworth PA, Blanshard JMV (1978) The interdependence of molecular structure and strength of carrageenan/carob gels Part I *Lebensm.-Wiss.u.-Technol.* 11: 279-282.

Ariga O, Takagi H, Nishizawa H, Sano Y (1987) Immobilization of microorganisms with PVA hardened by iterative freezing and thawing, *J. Ferment. Technol.* 65: 651-658.

Arnaud JP, Lacroix C, Choplin L (1989) Effect of lactic fermentation on the rheological properties of κ-carrageenan/locust bean gum mixed gels inoculated with *S. thermophilus*, *Biotech. Bioeng.* 34: 1403-1408.

Asano H Myoga H, Asano M, Toyao M (1992) Nitrification treatability of whole microorganisms immobilized by the PVA freezing method, *Wat. Sci. Tech.* 26: 2397-2400

Audet P, Paquin C, Lacroix C (1990) Batch fermentations with a mixed culture of lactic acid bacteria immobilized separately in κ-carrageenan/locust bean gum gel beads, *Appl. Microbiol. Biotechnol.* 32: 662-668.

Chamy R, Nunez MJ, Lema JM (1990) Optimization of the hardening treatment of *S. cereviseae* bioparticles, *Biotech. Bioeng.* 30: 52-59.

Chen K-C, Lin Y-F (1994) Immobilization of microorganisms with phosphorylated polyvinyl alcohol (PVA) gel, *Enzyme Microb. Technol.* 16: 79-83

De Gooijer CD, Wijffels RH, Tramper J (1991) Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in carrageenan. Part I: Dynamic modelling, *Biotech. Bioeng.* 38: 224-231.

Gotoh T, Unno H, Shiragami N, Honda H, Naoki Y (1993) Mass-production of biocatalyst-entrapping alginate gel particles by a forced oscillation method, *Chem. Eng. Comm.* 120: 73-84.

Hashimoto S, Furukawa K (1987) Immobilization of activated sludge by PVA-boric acid method, *Biotech. Bioeng.* 15: 52-59

Hulst AC, Tramper J, Van 't Riet K, Westerbeek JMM (1985) A new technique for the production of immobilized biocatalyst in large quantities, *Biotech. Bioeng.* 27: 870-876.

Hulst AC, Hens HJH, Buitelaar RM, Tramper J (1989) Determination of the effective diffusion coefficient of oxygen in gel materials in relation to gel concentration, *Biotech. Techn.* 3: 199-201.

Hunik JH, Tramper J (1993) Large-scale production of κ-carrageenan droplets for gel bead production: theoretical and practical limitations of size and production rate, *Biotech. Progress* 9: 186-192.

Hunik JH, Bos CG, Van den Hoogen MP, De Gooijer CD, Tramper J (1994a) Co-immobilized *Nitrosomonas europaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in kappa-carrageenan gel beads, *Biotech. Bioeng.* 43: 1153-1163.

Hunik JH, Tramper J, Wijffels RH (1994b) A strategy to scale-up nitrification processes with immobilized cells of *Nitrosomonas europaea* and *Nitrobacter agilis*. *Bioprocess Eng.* 11: 73-82.

Ichijo H, Nagasawa J, Yamauchi A (1990) Immobilization of biocatalysts with poly(vinyl alcohol) supports, *J. Biotech.* 14: 169-178

Klein J, Vorlop KD (1983) Immobilized cells-catalysts preparation and reaction performance, *ACS Symp. Ser.* 207: 377-392.

Krouwel PG (1982) Immobilized cells for solvent production. Ibe and ethanol fermentations, PhD-thesis Delft University.

Kurosawa H, Matsumura M, Tanaka H (1989) Oxygen diffusivity in gel beads containing viable cells, *Biotech. Bioeng.* 34: 926-932.

Leenen EJTM, Tramper J, Wijffels RH (1994) High rate nitrification with immobilized nitrifying bacteria, In: *ICHEME-Environmental Technology*, Brighton 4-6 july 1994: 52-54.

Lewandowski Z, Bakke R, Characklis WG (1987) Nitrification and autotrophic denitrification in calcium alginate beads, *Wat. Sci. Tech.* 19: 175-182.

Martinsen A, Skjåk-Bræk G, Smidsrød O (1989) Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads, *Biotech. Bioeng.* 33: 79-89.

Muscat A, Beyersdorff J and Vorlop KD (1993) Poly(carbamoylsulfonate), a material for immobilization: synthesis, diffusion- and mechanical properties, *Biotechnol. Techniques* 7: 591-596.

Myoga H Asano H, Nomura Y, Yoshida H (1991) Effects of immobilization conditions on the nitrification treatability of entrapped cell reactors using PVA freezing method, *Wat. Sci. Tech.* 23: 1117-1124

Ogbonna JC, Matsamura M, Yamagata T, Sakuma H, Kataoka H (1989) Production of micro-gel beads by a rotating disk atomizer, *J. Ferment. Bioeng.* 68: 40-48.

Østgaard K, Knutsen SH, Dyrset N, Aasen IM (1993a) Production and characterization of guluronate lyase from *Klebsiella pneumoniae* for applications in seaweed biotechnology, *Enzyme Microb. Technol.* 15: 756-763.

Østgaard K, Wangen BF, Knutsen SH, Aasen IM (1993b) Large-scale production and purification of κ-carrageenase from *Pseudomonas carrageenovora* for applications in seaweed biotechnology, *Enzyme Microb. Technol.* 15: 326-333.

Poncelet D, Lencki R, Beaulieu C, Halle JP, Neufeld RJ, Fournier A (1992) Production of alginate beads by emulsification/internal gelation. I. Methodology, *Appl. Microbiol. Biotechnol.* 38: 39-45.

Renneberg R, Sonomoto K, Katoh S and Tanaka A (1988) Oxygen diffusivity of synthetic gels derived from prepolymers, *Appl. Microbiol. Biotechnol.* 28: 1-7.

Shindo S, Kamikura M (1990) Immobilization of yeast with hollow PVA gel beads, *J. Ferment. Bioeng.* 70: 232-234.

Smidsrød O, Skjåk-Bræk G (1990) Alginate as immobilization matrix of cells, *Trends in Biotechnology* 8: 71-78.

Smidsrød O, Christensen BE (1991) Molecular structure and physical behaviour of seaweed colloids as compared with microbial polysaccharides. In: Seaweed resources in Europe: Uses and potential, Guiry MD, Blunden G (eds.), 185-217.

Stormo KE, Crawford RL (1992) Preparation of encapsulated microbial cells for environmental applications, *Appl. Environ. Microb.* 58: 727-730.

Sumino T, Nakamura H, Mori N, Kawaguchi Y, Tada M (1992[a]) Immobilization of nitrifying bacteria in porous pellets of urethane gel for removal of ammonium nitrogen from waste-water, *Appl. Microbiol. Biotechnol.* 36: 556-560.

Sumino T, Nakamura H, Mori N, Kawaguchi Y (1992[b]) Immobilization of nitrifying bacteria by polyethylene glycol prepolymer, *J. Ferment. Bioeng.* 73: 37-42.

Takeshima M, Kimata T, Mori N, Emori H (1993) "Pegasus", an innovative high-rate BOD and nitrogen removal process for municipal wastewater, 66th WEF Annual Confual Conference.

Tanaka H, Matsamura M, Veliky IA (1984) Diffusion characteristics of substrates in Ca-alginate gel beads, *Biotech. Bioeng.* 26: 53-58.

Tanaka K, Tada M, Kimata T, Harada S, Fujii Y, Mizuguchi T, Mori N, Emori H (1991) Development of new nitrogen removal systems using nitrifying bacteria immobilized in synthetic resin pellets, *Wat. Sci. Tech.* 23: 681-690.

Tramper J, Grootjen DRJ (1986) Operating performance of *Nitrobacter agilis* immobilized in carrageenan, *Enzyme Microb. Technol.* 8: 477-480.

Van Ginkel CG, Tramper J, Luyben KChAM, Klapwijk A (1983) Characterization of *Nitrosomonas europaea* immobilized in calcium alginate, *Enzyme Microb. Technol.* 5: 297-303.

Van Vliet T (1991a) Terminology to be used in cheese rheology, In: Rheology and fracture properties of cheese, *Bulletin of the international dairy federation* no. 268: 5-15.

Van Vliet T, Lucisano M, Casiraghi E (1991b) Inventory of test methods, In: Rheology and fracture properties of cheese, *Bulletin of the international dairy federation* no. 268: 16-25.

Vorlop K-D, Muscat A, Beyersdorff J (1992) Entrapment of microbial cells within polyurethane hydrogel beads with the advantage of low toxicity, *Biotech. Techn.* 6: 483-488.

Weast RC (1979) CRC Handbook of Chemistry and Physics. CRC Press Inc., Boca Raton, Florida, USA: F-62.

Wijffels RH, De Gooijer CD, Kortekaas S, Tramper J (1991) Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in carrageenan. Part II: Model evaluation. *Biotech. Bioeng.* 38: 232-240.

Wijffels RH, De Gooijer CD, Schepers AW, Beuling EE, Mallée L, Tramper J (1995[a]) Dynamic modelling of immobilized *Nitrosomonas europaea*: implementation of diffusion limitation over expanding micro-colonies. *Enzyme Microb. Technol.*, 17: 462-471.

Wijffels RH, Englund G, Hunik JH, Leenen EJTM, Bakketun Å, G_nther A, Obón de Castro JM, Tramper J (1995b) Effect of diffusion limitation on immobilized nitrifying microorganisms at low temperatures. *Biotech. Bioeng.*, 45: 1-9.

Wijffels RH, Tramper J (1995) Nitrification by immobilized cells, *Enzyme Microb. Technol.*, 17: 482-492.

Willke B, Willke T, Vorlop K-D (1994) Poly(carbamoylsulphonate) as a matrix for whole cell immobilization - Biological characterization, *Biotech. Techn.* 8: 623-626.

Willke Th, Vorlop KD (1995) Nitrification in PVAL-beads: Influence of pH and temperature on nitrite oxidation, Proc. 7th European Congress on Biotechnology.

Wise DL, Houghton G (1966) The diffusion coefficients of ten slightly soluble gases in water at 10-60°C. *Chem. Eng. Sci.* 21: 999-1010.

Wu K-YA, Wisecarver KD (1992) Cell immobilization using PVA crosslinked with boric acid, *Biotech. Bioeng.* 39: 447-449.

This chapter has been published in: Biotechnology Progress, 1996, 12:163-171by Martins dos Santos VAP, Marchal L, Tramper J, Wijffels RH.

## CHAPTER 7

# Modeling and Evaluation of an Integrated Nitrogen Removal System with Microorganisms Co-immobilized in Double-Layer Gel Beads


## ABSTRACT

A dynamic model describing integrated nitrification and denitrification by *Nitrosomonas europaea and Pseudomonas* spp. co-immobilized in the separate layers of doublelayer gel beads is presented. The model describes diffusion of components, substrate utilization, and growth, all occurring simultaneously in the beads. Both internal and external mass transfer resistance are accounted for. The model predicts biomass and solute bulk concentrations, substrate consumption rates, product formation rates, and biomass and solute concentration profiles within the beads as a function of time. Fluctuations in substrate load, dilution rates, or mass transfer parameters can be accommodated as well. Intrinsic kinetic parameters of the microorganisms, internal and external mass transfer coefficients, initial conditions, bead concentration, and particle diameters are the input parameters. The model was evaluated by comparing experimental and predicted bulk concentrations and macroscopic consumption (production) rates in air-lift loop reactors containing double-layer gel beads. The reactors were run under both steady and dynamic operating conditions. Nitrification rates were determined by daily analysis of influent and effluent ammonia and nitrite concentrations, and denitrification rates were calculated from molecular nitrogen production rates measured by headspace analysis. Model predictions agreed reasonably well with the experimental results. Fluctuations in dilution rates and influent substrate concentrations were adequately described. Although further validation is still required, the model presented here has shown to describe satisfactorily the proposed system.

## INTRODUCTION

An integrated system for nitrogen removal with pure strains of nitrifying and denitrifying cells co-immobilized in double-layer gel beads has been proposed earlier (Tramper, 1984; Martins dos Santos *et al.,* 1993, 1996a). It has been shown that, by making use of the simultaneous coexistence of aerobic and anoxic zones within the support, coupled nitrification and denitrification can indeed be achieved in an air-lift reactor with co-immobilized *Nitrosomonas europaea* and either *Pseudomonas* spp. or *Paracoccus denitrificans* (Martins dos Santos *et al.,* 1996a). In this way, oxygen diffusion limitation is used as a benefit rather than a disadvantage as it provides a suitable environment for the (facultative) anaerobic denitrifier. Therefore, this two-stage process can be conducted as if single-staged. Mathematical modeling of the (dynamic) behavior of the system is an important tool to evaluate its potential applications. Moreover, the knowledge and insight gained can be of considerable importance in the study of immobilized co-culture systems for the integration of oxidative and reductive biodegradation processes in general. Examples could be, for instance, the biodegradation of aromatic pollutants or other recalcitrant compounds such as DDT (Beunink and Rehm, 1988), 4-chloro-2-nitrophenol (Beunink and Rehm, 1990), 2,4,6trichlorophenol (Gardin and Pauss, 1994), 2,3,6-trichlorobenzoic acid (Gerritse and Gottschal, 1992), and polychlorinated hydrocarbons (Fathepure and Vogel, 1991), which are known to require both aerobic and anaerobic steps to be fully mineralized.

In this article, a dynamic model for the above-mentioned system, considering simultaneous mass transfer, substrate consumption, and growth, is presented and evaluated experimentally at reactor level. Both external and internal mass transfer limitations are accounted for. A schematic representation of the system is given in Figure 1. In the outer layer, *Ns. europaea* converts aerobically ammonium into nitrite, and this is subsequently (anaerobically) reduced to molecular nitrogen by *Pseudomonas* spp. *or Pa. denitrificans* with simultaneous consumption of acetate. The model predicts reactor bulk concentrations and biomass and solute concentration profiles within the beads as function of time. Input parameters are intrinsic kinetic parameters of the microor-aanisms, internal-diffusion coefficients, and masstransfer coefficients. These parameters are taken from the literature. All substrates, i.e. $NH_4^+$, $NO_2^-$, $O_2$, and acetate can be, in principle, growth limiting. The model can handle time fluctuations in the dilution rate, substrate loading, and mass-transfer parameters. The model was evaluated experimentally by measuring the macroscopic substrate consumption and product formation rates. For that, two air-lift loop reactors containing either 15 or 25% (v/v) double-layer beads were independently operated at constant and variable operating

conditions, respectively. Reactor bulk concentrations of nitrogen compounds and nitrogenous-gas production were measured daily. Predicted and measured effluent concentrations agreed reasonably well with each other. The model accounted adequately for changes in the influent substrate concentrations and dilution rates.

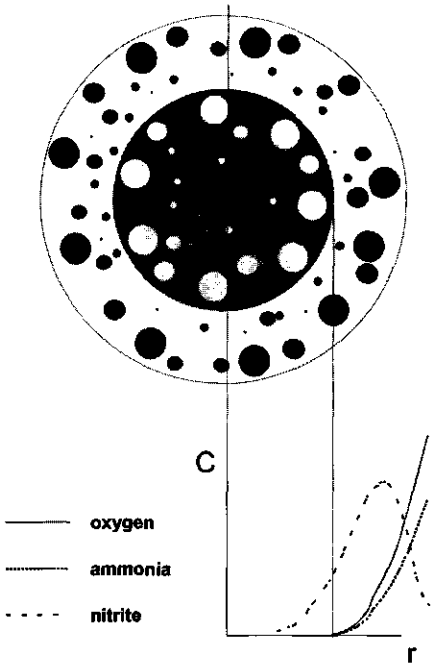


Figure 1. Schematic representation of the "Magic Bead Concept". In the outer layer nitrification occurs. In the core denitrification takes place.

## MODEL DEVELOPMENT

### Stoichiometry

The following stoichiometric reactions are used in the modelling. These equations are based on elemental balances both for substrate conversion and synthesis of biomass. For nitrification by *Nitrosomonas europaea* the energy-yielding reaction (outer layer) comes as:

$$NH_4^+ + 1.5\,O_2 \Rightarrow NO_2^- + H_2O + 2\,H^+ \qquad (a)$$

Growth of *Nitrosomonas europaea*, using the empirical cell composition $C_5H_7NO_2$

(EPA, 1975) is described as:

$$13\ NH_4^+ + 15\ CO_2 \Rightarrow 10\ NO_2^- + 3\ C_5H_7NO_2 + 4\ H_2O + 23\ H^+ \quad (b)$$

The equations have terms showing the production of free acid and consumption of gaseous carbon dioxide. These reactions take place preferably at pH values between 6.5 and 8.5 (convenient pH range for the microorganisms involved). Under these circumstances, the production of acid results in immediate reaction with $HCO_3^-$ to form $H_2CO_3$ ($K_a = 4.45 * 10^{-7}$ mol.l$^{-1}$, at 25°C).

The equation for the energy-yielding reaction can be combined with the equation for biomass synthesis into an overall energy/synthesis relation by the true yield coefficient of *Nitrosomonas europaea* on ammonia ($2.163 * 10^{-3}$ $kg_{biomass}$· mol $N^{-1}$, see section of Input parameters). The overall equation (accounting already for the changes in the carbonic-acid system) becomes thus:

$$NH_4^+ + 1.379\ O_2 + 1.986\ HCO_3^- \Rightarrow$$
$$0.983\ NO_2^- + 0.0191\ C_5H_7NO_2 + 1.041\ H_2O + 1.890\ H_2CO_3 \quad (c)$$

For *Pseudomonas* spp. an analogous procedure was followed to obtain the following overall energy/synthesis equation for denitrification:

$$NO_2^- + 0.0654\ NH_4^+ + 0.537\ CH_3COOH + 0.184\ H_2CO_3 \Rightarrow$$
$$0.5\ N_2 + 0.0654\ C_5H_7NO_2 + 0.934\ HCO_3^- + 0.696\ H_2O \quad (d)$$

*Pseudomonas* spp. is a facultative heterotrophic organism and therefore in the presence of oxygen acetate is oxidized as follows (overall equation):

$$CH_3COOH + 0.122\ NH_4^+ + 1.393\ O_2 + 0.1215\ HCO_3^- \Rightarrow$$
$$1.514\ H_2CO_3 + 0.122\ C_5H_7NO_2 + 0.364\ H_2O \quad (e)$$

**Model Description**

Substrate consumption and biomass production rates are described by the method introduced by Beeftink *et al.* (1990) and used by Hunik *et* al. (1994), de Gooijer *et al.*

(1991), and Wijffels *et al.* (1991, 1993, and 1995). The pertinent equations account for negative net growth (cell death) at low substrate concentrations and an observable maximum growth rate at nonlimiting conditions (see eqs 2 and 3, Appendix). Cell density is limited by a maximum observable biomass concentration in the support ($X^m_{max}$). For the nitrifying cells in the outer envelope, the surplus of biomass is excreted into the bulk, as earlier described (Wijffels *et al.*, 1994, 1995; Willaert, 1993). Substrate consumption by the released cells is accounted for in the model [eq 6, Appendix], but their contribution is small since the dilution rate in the reactor is well above the maximum specific growth rates of the microorganisms involved. Since there is no transport of biomass within the support, is assumed that the *Pseudomonas* spp. cells remain at their maximum attainable concentration and that substrate utilization proceeds accordin to eq 2. Mass transport is described by the equations for diffusion within the particles and for external mass-transfer resistance between bulk and gel bead. The biocatalytic particle consists of a gel core surrounded by a gel envelope. The gel materials can be different, as well as the respective diffusion coefficients for solutes in the gels. We assumed, however, that these coefficients are the same for both layers. It is further assumed that there is no gap between the two matrices and that the partition coefficient of the solutes is 1. In the model, the gel bead is divided in 244 equally spaced shells and uniform conditions within each shell are assumed.

The input parameters include substrate influent concentrations, dilution rate, bead holdup, bulk oxygen concentration, and initial biomass concentrations. The model accounts for fluctuations in these parameters, but in the simulations, only acetate and ammonium concentrations and the dilution rates were varied in time. The output consists of bulk biomass, substrate and product concentrations, their respective profiles across the gel beads, and macroscopic nitrogen production rates.

**Criteria for the "Most-Limiting" Substrate**

The choice of the "most-limiting" substrate is based on a noninteractive model for growth under double-substrate limitation (Ryder and Sinclair, 1972; Bader, 1978; Mankad and Nauman, 1992). According to this model, the specific growth rate of a microorganism is limited by a single substrate at a time. The growth rate of the organism would equal the lowest value predicted from separate single-substrate models (eq 1, Appendix). In our system this means that, within a given shell, if two substrates are in theory limiting, the actual substrate conversion will be governed by the "most-limiting" substrate, which is the one with the lowest "Monod term". In the model, the limiting substrates are determined for each shell at every time step. Since *Pseudomonas*

spp. is a facultative heterotrophic microorganism (Robertson *et al.*, 1989), an additional criterium is required to decide whether nitrite or oxygen is used as electron acceptor, which obviously determines growth and conversion rates. Although denitrification is most efficient under anoxic conditions, it has been shown that this *Pseudomonas* strain denitrifies up to air saturation of 70-80% (Robertson and Kuenen, 1990; Robertson *et al.*, 1989). Within this range the denitrifying capacity varies widely but tends to decrease with an increase in oxygen concentration. We assumed a "threshold oxygen tension" value of 30%, below which mainly denitrification occurs if nitrite is present in sufficient amounts (L. Robertson, personal communication). Hence, within a given shell, nitrite is used if the local oxygen tension is lower than the threshold value and if the Monod term for nitrite is larger than for oxygen. Otherwise oxygen is used. After that, the Monod terms for the electron acceptor and for acetate are compared to determine the "most-limiting" substrate. This choice will affect the biomass production rate as well since the respective specific growth rates are different in the presence and absence of oxygen. After choosing the "most-limiting" substrate, the conversion of the nonlimiting substrates is calculated using the Monod term for the limiting substrate in eq 2. Substrate and product inhibition for *Ns. europaea* are unimportant for ammonia concentrations up to 100 moi.m$^{-3}$ and up to 50 mol.m$^{-3}$ for nitrite, respectively (Hunik *et al.*, 1992). Within the relatively low concentration ranges used, it is unlikely that nitrite inhibition would occur for *Pseudomonas* spp. either (Wang *et* al., 1995; Almeida *et al.*, 1995).

**Calculation procedures.**

The calculation procedures were similar to the ones used by Hunik *et al.* (1994). The ordinary differential eq 3 (Appendix) for biomass growth in the gel beads was solved numerically with a second order Runge-Kutta algorithm. The equations for the bulk concentrations of biomass (eq 5) and substrate (eq 6) were solved with an algorithm based on the trapezoidal rule. The substrate concentration profiles in the gel beads were calculated using a backward-in-time and centered-in-space algorithm for partial differential equations. This finite-differences method allows changes in limiting substrate at any place in the gel bead and is, in general, unconditionally stable (Press *et al.*, 1989).

A time-step size of 50 s was chosen as it gives a sufficiently accurate solution in a reasonable computation time. An average simulation run for 30 days takes about 15 min of CPU time on an IBM-compatible computer with a pentium processor (100 MHz).

Table I - Model Parameters

| Parameter | Value | Dimensions | Parameter | Value | Dimensions |
|---|---|---|---|---|---|
| $\mu_{max,O2}^{Ps}$ | $2.78 \times 10^{-5}$ | $s^{-1}$ | $X^{Ps}$ | 170 | $kg.m^{-3}$ |
| $\mu_{max,NO2}^{Ps}$ | $3.33 \times 10^{-5}$ | $s^{-1}$ | $K_{I,s}^{NH4}$ | $2.21 \times 10^{-5}$ | $m.s^{-1}$ |
| $\mu_{max}^{Ns}$ | $1.52 \times 10^{-5}$ | $s^{-1}$ | $K_{I,s}^{NO2}$ | $2.21 \times 10^{-5}$ | $m.s^{-1}$ |
| $m_{O2}^{Ns}$ | $9.4 \times 10^{-4}$ | mol $O_2.kg^{-1}.s^{-1}$ | $K_{I,s}^{O2}$ | $2.63 \times 10^{-5}$ | $m.s^{-1}$ |
| $m_{NO2}^{Ps}$ | $5.44 \times 10^{-4}$ | mol $NO_2.kg^{-1}.s^{-1}$ | $K_{I,s}^{N2}$ | $2.63 \times 10^{-5}$ | $m.s^{-1}$ |
| $m_{O2}^{Ps}$ | $3.84 \times 10^{-4}$ | mol HAc.$kg^{-1}.s^{-1}$ | $K_{I,s}^{HAC}$ | $2.07 \times 10^{-5}$ | $m.s^{-1}$ |
| $Y_{NH4}^{Ns}$ | $1,66 \times 10^{-3}$ | $kg.mol^{-1}$ $NH_4$ | $D_w^{NH4}$ | $2.20 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{O2}^{Ns}$ | $1.19 \times 10^{-3}$ | $kg.mol^{-1}$ $O_2$ | $D_w^{NO2}$ | $2.20 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{O2}^{Ps}$ | $9.86 \times 10^{-3}$ | $kg.mol^{-1}$ $O_2$ | $D_w^{N2}$ | $2.83 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{NO2}^{Ps}$ | $7.39 \times 10^{-3}$ | $kg.mol^{-1}$ $NO_2$ | $D_w^{O2}$ | $2.83 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{HAC}^{Ps}$ | $1.37 \times 10^{-2}$ | $kg.mol^{-1}$ HAc* | $D_w^{HAC}$ | $2.00 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{O2}^{Ns}$ | $5.05 \times 10^{-3}$ | $mol.m^{-3}$ | $D_g^{NH4}$ | $1.78 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{NH4}^{Ns}$ | 1,25 | $mol.m^{-3}$ | $D_g^{NO2}$ | $1.78 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{O2}^{Ps}$ | $2 \times 10^{-3}$ | $mol.m^{-3}$ | $D_g^{N2}$ | $2.30 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{NO2}^{Ps}$ | 0,31 | $mol.m^{-3}$ | $D_g^{O2}$ | $2.30 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{HAC}^{Ps}$ | 0,73 | $mol.m^{-3}$ | $D_g^{HAC}$ | $1.62 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $X^{Ps}$ | 36,7 | $kg.m^{-3}$ | * HAc – Acetate | | |

**Input parameters**

***Transport parameters***

The diffusion coefficients for $NH_4^+$ and $NO_2^-$ in carrageenan and alginate at 30 °C are the same as used by Hunik *et al.* (1994), who reviewed and evaluated literature data on diffusion coefficients for nitrogen ionic compounds. These are in average 80% of their value in water (Table I). Similarly, the effective diffusion coefficient for acetate was assumed to be 80 % of its value ($2.83 * 10^{-9}$ $m.s^{-1}$) in water at 30°C (Perry & Green, 1984). For oxygen, we used an average diffusion coefficient of $2.05 * 10^{-9}$ $m.s^{-1}$ (Hunik *et al.*, 1994). The same value is taken for the diffusion coefficient of molecular nitrogen in the gels. The liquid/solid mass-transfer coefficients are estimated using the relation of Ranz and Marshall, as previously done (Wijffels *et al*, 1991, Hunik *et al.*, 1994).

***Biological parameters***

The *Nitrosomonas europaea* cells are from the same continuous culture as used by Hunik *et al.* (1994,1992) and Wijffels *et al* (1994, 1995). Therefore, the biological parameters used in the model are the same as the ones used by these authors (Table I). The yield of *Nitrosomonas* on ammonia includes an increase of 30% in relation to that

measured by Hunik *et al* (1994) due to the presence of acetate, such as reported by Van Niel (1991). The *Pseudomonas* spp. strain was the same as the one used by Robertson and co-workers (Robertson *et al.*, 1989a,b, 1990, van Niel *et al.*, 1991,1993). We have used their kinetic parameters to estimate the input values in our model as they present parameters on nitrate but not on nitrite. Robertson *et al.* (1989), give an observed yield on acetate at different growth rates, oxygen concentrations and nitrate concentrations. From these data a true yield on acetate (without oxygen and with nitrate) was calculated by plotting the reciprocal of the observed yield against the inverse of growth rate (Koike & Hattori, 1975). This true yield was then used to couple growth and consumption (based on nitrate) equations into an overall reaction equation in a similar way as done for *Ns. europaea*. From the overall energy/synthesis equation obtained a true yield of biomass on nitrate was calculated. Assuming that the ratio of the yields on nitrite and nitrate is proportional to the energy ratio (redox potential) of nitrite and nitrate (as suggested by Zumft (1991), and Koike & Hattori (1975)), a yield of biomass on nitrite was calculated. Using this value, an overall energy/synthesis equation with nitrite [Eq. (d)] was developed as done above. The yield on acetate under fully aerobic conditions and in absence of nitrite was taken directly from data of Robertson *et al* (1989), as well as the maintenance coefficients. For both strains, the maintenance coefficients on each substrate were related to each other according to their stoichiometric proportions. The maximum biomass concentration for *Nitrosomonas europaea* was taken as 36.6 $kg.m^{-3}_{gel}$ (dry weight) as previously measured by Wijffels *et al* (1994). For *Pseudomonas* sp., a value of 170 $kg.m^{-3}$ was used according estimates of Gujer & Boller (1989) for heterotrophic bacteria in a biofilm.

## EXPERIMENTAL METHODS

### Strains and Media

*Nitrosomonas europaea* ATCC 19718 and *Pseudomonas* spp., strain LMD 84.60 (formerly designated as *Pseudomonas denitrificans*) were cultivated, harvested and prepared for immobilization in the same way as described before (Martins dos Santos *et al.*, 1996).

### Immobilized-cell concentration.

Estimates of the initial biomass concentration were derived from activity assays in a biological oxygen monitor as described before for nitrifying cells (Van Ginkel *et al.*, 1983). In a reaction cuvette 3 ml potassium phosphate buffer (0.1 $mol.dm^{-3}$, pH 7.8) were mixed with 1 ml of the cell suspension used for immobilization. The mixture was

aerated for about 15 minutes until air-saturation was obtained. Through a small opening a concentrated substrate solution was injected so that the final substrate concentration was never limiting and the decrease in oxygen concentration at 30°C was recorded. The substrates for *Nitrosomonas europaea* and *Pseudomonas* spp. were $NH_4Cl$ and $NaCH_3COO$, respectively. The volumetric activity was converted to a biomass dry-weight concentration by dividing by the specific activity of the microorganism. The specific activity values are based on the maximum specific growth rate, yield of biomass on substrate and maintenance coefficients (see Input parameters) combined according to Eq.2 (Appendix). By filling in Eq. 2 for both strains, the specific activity values were calculated as being $1.06 * 10^{-2}$ $mol.kg^{-1}.s^{-1}$ for *Ns. europaea* and $3.20 * 10^{-3}$ $mol.kg^{-1}.s^{-1}$ for *Pseudomonas* spp., respectively.

**Immobilized-cell cultivation**

*Nitrosomonas europaea* and *Pseudomonas* sp. cells were co-immobilized in the carrageenan and alginate layers, respectively, of double-layers beads obtained by encapsulation. The immobillization conditions and entrapment procedure were those described elsewhere (Martins dos Santos *et al.*, 1995 a,b). The co-immobilized cells were cultivated in two continuous internal air-lift loop reactors (A and B) under aseptic conditions. Beads used in reactor A had a core with a radius of $1.4 * 10^{-3}$ m and a total radius of $2.51 * 10^{-3}$ m. The initial biomass concentrations of *Nitrosomonas europaea* and *Pseudomonas* spp. were $2.76 * 10^{-3}$ and $1.62 * 10^{-2}$ $kg.m^{-3}_{gel}$, respectively. The average core and total radii of the beads used in reactor B were 1.23 and $2.1 * 10^{-3}$ m, respectively. Initial biomass concentrations were $4.29 * 10^{-3}$ $kg.m^{-3}_{gel}$ for *Ns. europaea* and $1.15 * 10^{-2}$ $kg.m^{-3}_{gel}$ for *Pseudomonas* spp. The two reactors A and B had a working volume of 440 ml and 385 ml and a headspace volume of 540 ml and 230 ml, respectively. The reactors were loaded with 15 and 25 % (v/v) beads, respectively. Cultivation medium contained the following (per litre): 10 mmol KCl, 0.4 mmol $MgSO_4$, 5.0 mmol Tris, 0.5 mmol $KH_2PO_4$, 2.1 mmol $CaCl_2$ and 2 ml of Vishniac & Santer trace-elements solution (Vishniac & Santer, 1957). The medium was sterilized by cold filtration using a Sartobran PH Capsule, pore size 0.2 µm (Sartorius AG, Göttingen Germany). Reactor A was continuously supplied with 13 mM acetate and 18 mM ammonium, while to reactor B variable concentrations of $CH_3COOK$ (5 - 10 mM) and $NH_4Cl$ ( 5 -12 mM) were added. Temperature was kept at 30 °C and the pH controlled (LH Fermentation 500 Series III) to 7.5 with 0.25 M HCl and 0.25 M KOH. The pH-

control solutions were filter sterilized using a Millex $FG_{50}$ 0.2 mm Filter unit (Millipore S.A. Molsheim, France). Influent and effluent samples were taken daily for analysis of acetate and nitrogenous compounds. Air was supplied at a superficial velocity of about $3.5 * 10^{-2}$ $m.s^{-1}$. Reactor A was run with a constant feed at a dilution rate of $1.8 * 10^{-4}$ $s^{-1}$ (working volume basis). Reactor B was run for 21 days with variable dilution rate and variable influent concentrations of both ammonia and acetate. The dilution rate was reduced from $6.67 * 10^{-4}$ $s^{-1}$ to $3.78 * 10^{-4}$ $s^{-1}$ and then to $1.89 * 10^{-4}$ $s^{-1}$ eigth and fifteen days after the start up, respectively. The influent substrate concentrations were continuously varied between 5 and 12 mM for ammonia and between 5 and 10 mM for acetate. Headspace analysis was executed daily for measurement of the production rate of nitrogenous gases and the consumption rate of oxygen. This was done in the same way as previously described (Martins dos Santos *et al.*, 1996).

**Analytical methods**

Ammonia, nitrite and nitrate (as control) concentrations in the influent and effluent of the continuous reactors were measured using a continuous-flow analysis system (Skalar Methods) as earlier described (Hunik *et al*, 1994). Influent and effluent acetate concentrations were measured by gas chromatography. The gas phase of the reactor head-space was analyzed (daily) by gas chromatography as well. Both measurements have been described before (Martins dos Santos *et al*, 1996).

## RESULTS AND DISCUSSION

**Reactor performance**

The model predicts time-dependent solute and biomass concentration profiles across the beads. From these, substrate and product concentrations in the reactor bulk are calculated. In this paper, we evaluate the model at reactor level. Therefore, predicted and measured bulk concentrations are compared both for constant and varying operating conditions (reactor A and B, respectively). In the first experiment, reactor A was continuously operated at a constant dilution rate of $1.8 * 10^{-4}$ $s^{-1}$. Figure 2 shows the measured and predicted effluent concentrations for ammonium (2a), nitrite (2b) and acetate (2c). The experimental ammonium concentrations agree well with those predicted (Figure 2a). Nitrification rates were calculated on the basis of the dilution rate and influent and effluent concentrations, correcting for fixation into biomass. After 7 days, a maximum ammonium conversion of 5.4 $mmol.m^{-3}_{gel}.s^{-1}$ was reached.

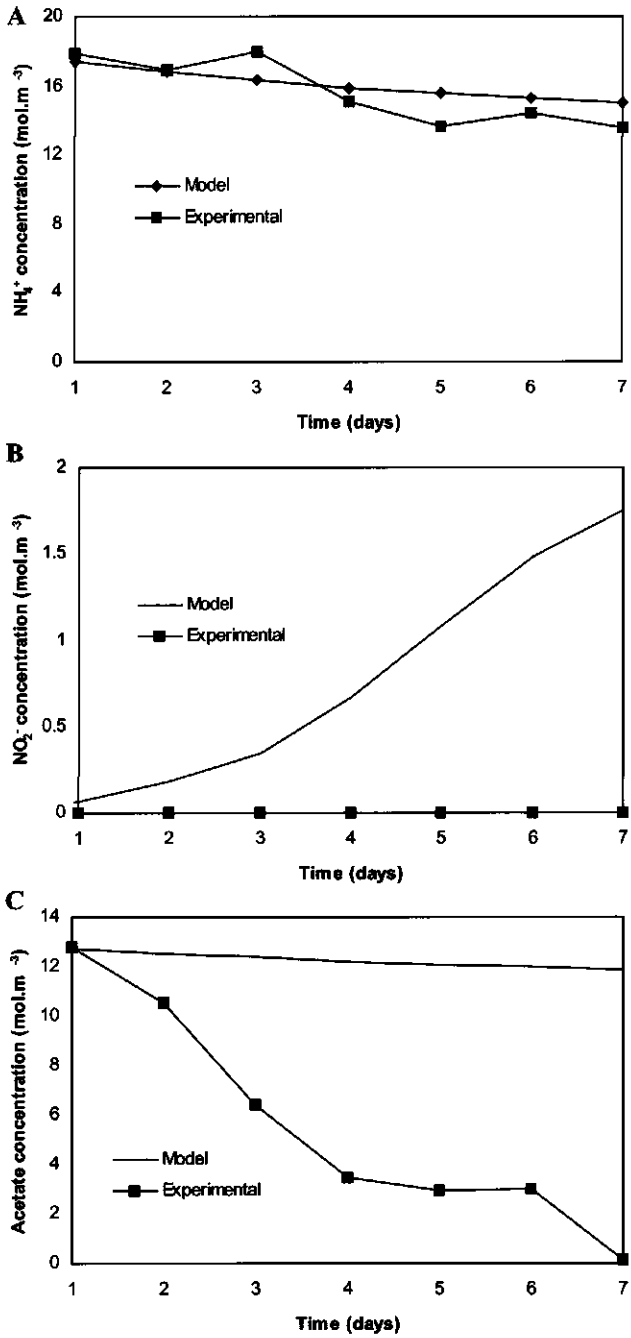


Figure 2 – Experimental and predicted concentrations in reactor A (constant operating conditions): a) ammonium; b) nitrite; c) acetate.

This is comparable to what we previously reported for the same system (Martins dos Santos *et al.*, 1995a) and is higher than reported for nitrification alone with immobilized cells (Wijffels *et al*, 1989, Hunik *et al*, 1994, van Ginkel *et al*, 1983). Yet, no nitrite was found in the effluent (Figure 2b), though some $NO_2^-$ would be expected to be present in the reactor outlet. According to the model, ammonia is oxidized mainly in the outer nitrifying layers and therefore the nitrite produced would diffuse both inwards and outwards (see profiles in Figure 1). The model predicts concentrations in the bulk up to 1.7 $mol.m^{-3}$ towards the end of the run (Figure 2b). Also, though theoretically only a very moderate reduction of the acetate concentration in the bulk is predicted, experimental effluent acetate concentrations rapidly decreased to very low values following the first days after start up (Figure 2c). A possible explanation for both deviations is given in the next section.

In a second experiment, the operational conditions were varied to check whether the model was able to describe the dynamic behaviour of the system. In this reactor (B), the dilution rate was reduced from 6.67 $*10^{-4}$ $s^{-1}$ to 3.78 $*10^{-4}$ $s^{-1}$ on the $8^{th}$ day after start up and to 1.89 $*10^{-4}$ $s^{-1}$ on the $15^{th}$. Both the ammonia and acetate concentrations in the influent were varied with time as shown in Figure 3a. These influent concentrations were introduced in the model after fitting the concentrations measured in the reactor inlet. The measured effluent concentrations of ammonia agreed well with the predicted values until day 13, after which they begin to differ. The nitrification rates determined on the basis of the experimental effluent concentrations were, however, somewhat lower than the predicted values during the whole run (Figure 3b). Similar results on nitrification with immobilized *Nitrosomonas europaea* have been reported by Wijffels *et al.* (1995).

To describe growth and substrate consumption in an air-lift reactor, they used a pseudo-homogeneous growth model that assumes no diffusion-limitation within the colonies. This led to overestimations of up to 50% of the macroscopic consumption rates. When diffusion limitation within the colonies was accounted for in the model (colony-expansion model) the experimental results were much better described. In another study, Wijffels and co-workers (1994) have shown that such diffusion limitation phenomena (and therefore reduced conversion rates) were indeed very likely to occur within colonies of immobilized *Nitrososmonas europaea* when low inoculum sizes were used.

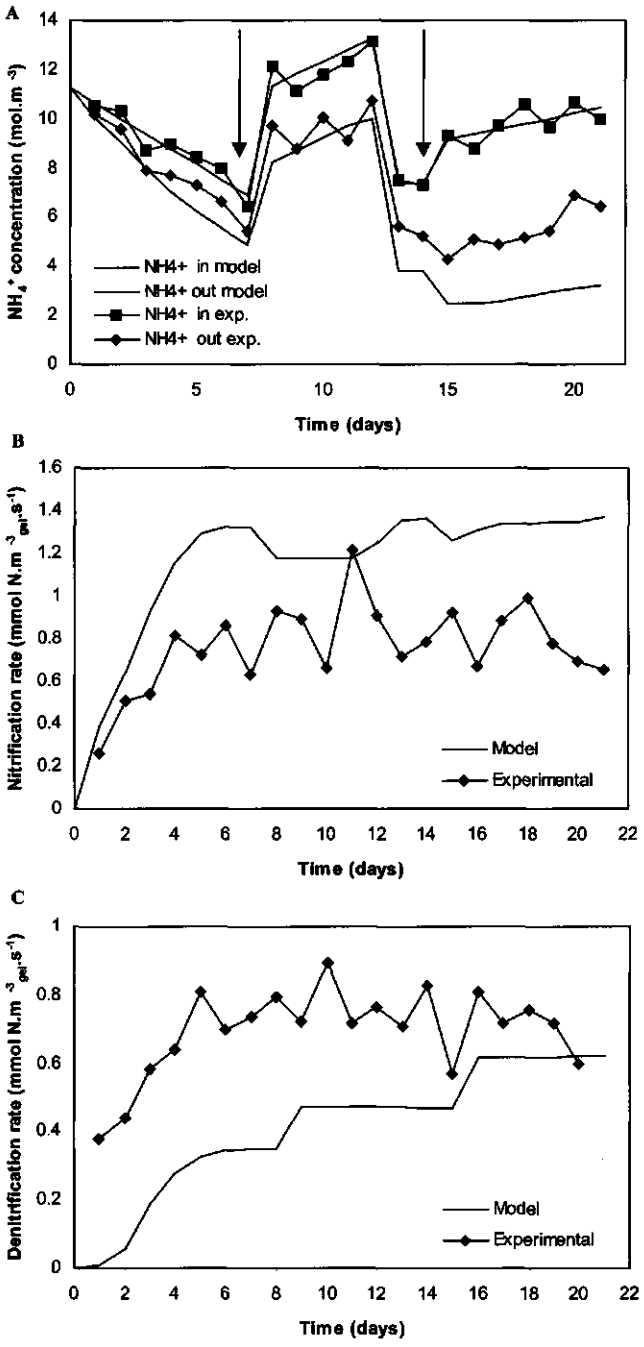


Figure 3 – Experimental versus model results of experiment B (variable operating conditions): (a) influent and effluent concentrations; b) nitrification; c) denitrification rate. The arrows indicate changes in the dilution rate.

Calculations showed that these effects could be neglected at inoculum sizes larger than 0.5 $kg.m^{-3}$. In the experiments A and B described in this paper, the initial biomass concentrations were more than two orders of magnitude lower than this value. It is thus very likely that the relatively low inoculum sizes would explain the deviations between predicted and measured nitrification rates.

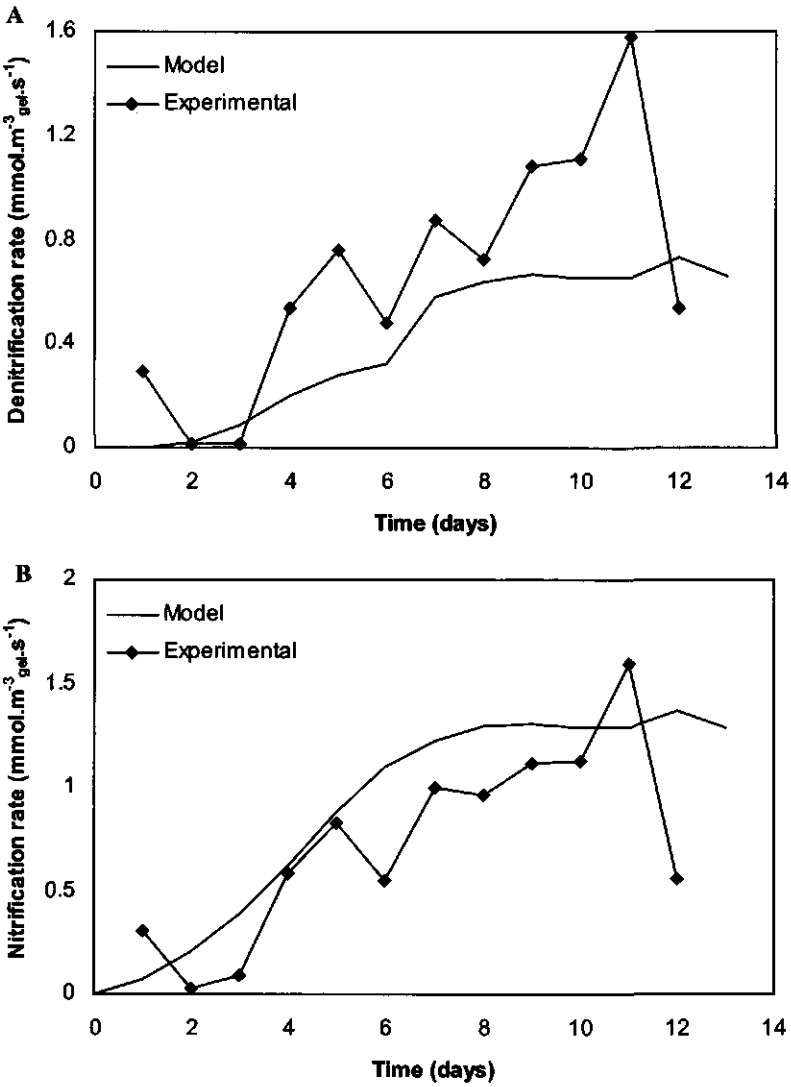


Figure 4 – Predicted and measured rates of nitrification (a) and denitrification (b) in previously reported experiments (Martins dos Santos *et al.* (1996)).

In contrast to nitrification, the actual denitrification rates determined on basis of the rate of gas production (headspace analysis) were well above the model predictions, specially during the first half of the run (Figure 3c). A possible explanation for this discrepancy will be discussed in the next section.

Although over and under estimated, respectively, the experimental nitrification and denitrification rates (expressed on N basis) were very close to each other during the whole run. This indicates that the ammonia used was fully mineralized to nitrogenous-gas compounds, as the nitrogen fixed into biomass was estimated to be only about 1.6 and 6 % of the total ammonia converted for *Nitrosomonas europaea,* and *Pseudomonas* sp., respectively (Martins dos Santos *et al.,* 1996). Similar to experiment A, practically no nitrite was found in the outlet of reactor B and the acetate present in the effluent rapidly decreased to negligible levels after the first 7 days of operation (not shown).

As further evaluation, model simulations were done for an earlier experiment with co-immobilized *Nitrosomonas europaea* and *Pseudomonas* spp. (Martins dos Santos *et al.*, 1996). The predicted and measured nitrification and denitrification rates are presented in the Figures 4a and 4b, respectively. The calculated nitrification rates agree relatively well with the experimental ones, while the denitrifying rates are, again, somewhat underestimated. As in reactors A and B, nitrite was not detected in the reactor effluent and the acetate decreased rapidly to negligible values.

**Nitrogen and acetate balances**

As mentioned above, in both reactors A and B almost no nitrite was found in the effluent, except for small peaks in run B following the changes in influent substrate concentrations and dilution rates. Analogously, in both experiments, the acetate levels in the reactor decreased rapidly and eventually reached zero after few days of operation. According to the model much less acetate should be used for denitrification and fixation into biomass (see Figure 2c for experiment A).

A possible cause of these effects could be the growth of *Pseudomonas* sp. outside the beads. Indeed, after a few days of operation a thin biofilm was visible on the reactor walls and on other surfaces in both reactors. In both experiments, a few beads that had lost their coatings could be observed. Very likely, these damaged beads were the source of suspended *Pseudomonas* cells in the reactor, which could then attach to the reactor surfaces. An estimate for "external" biomass (*Pseudomonas* spp.) density was made on the basis of the extra acetate consumed in the reactor A. The rate of extra acetate consumed for each day was filled in the equation for substrate consumption (Eq.[2], appendix). From there a biomass concentration was calculated for each day. From these

calculations it appears that "external" *Pseudomonas* at increasing concentrations up to 0.8 - 1.0 $kg.m^{-3}_{reactor}$ could be present in the reactor.

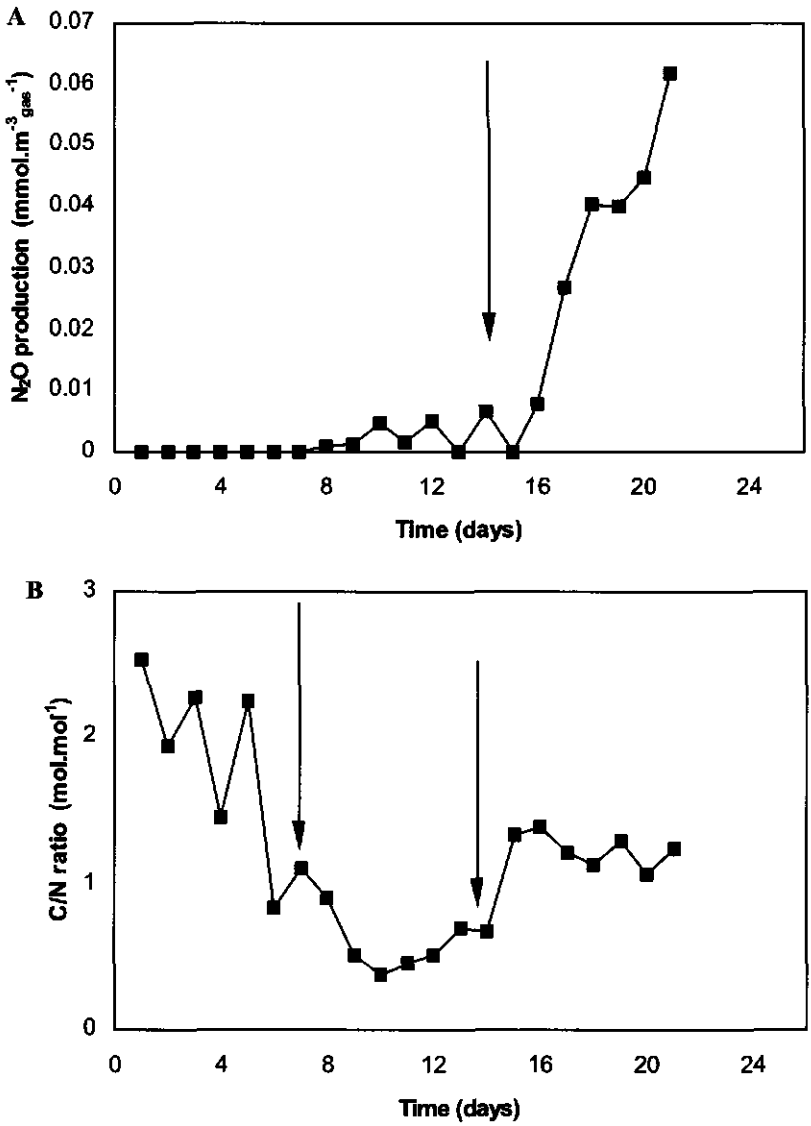


Figure 5 – Nitrous oxide production (a) and carbon-to-nitrogen ration in the influent of reactor B (b). The arrows indicate changes in the dilution rate.

Since the specific area of the reactor walls is about 160 $m^2$ $m^{-3}$, a biofilm density of about 5 - 6 * $10^{-6}$ $m^3_{cells}$ $m^{-2}$ $_{reactor}$ was calculated. This means that a very thin cell layer (about 5 mm thick) could be indeed responsible for the acetate losses in the system.

As stated above, this denitrifying strain we used has been reported to denitrify considerably under aerobic conditions (Robertson *et al*, 1990, Robertson and Kuenen, 1990). It is thus possible that the nitrite eventually released into the bulk would have been immediately consumed by the attached biomass resulting in enhanced denitrification rates. Mass balances for nitrogen, indicate that "nitrite losses" could indeed cover the extra production rates of nitrogenous gases. Accounting as well for nitrogen fixed into "external" *Pseudomonas* spp., element-nitrogen losses were negligible in both experiments.

**Nitrous oxide production**

In reactor B, the nitrogen production rate stabilized after 6-7 days at a level of about 0.77 $mmol.m^{-3}_{reactor}.s^{-1}$ and until then no nitrous oxide was detected (Figure 5a). At day 8 and 15, the reactor dilution rates were subsequently decreased and the influent acetate and ammonia concentrations were changed. Apparently, this led to a certain instability of the system, particularly after the second change. After the changes at day 15, the $N_2$ production rate progressively decreased and nitrous-oxide production augmented very rapidly, reaching about 14 % of the total N-gas produced towards the end of the experiment (Figure 5a). A comparable increase in the production rate of carbon dioxide was detected as well (data not shown). These two sudden increases followed a two-fold rise in the carbon-to-nitrogen ratio (mol/mol) in the influent load (Figure 5b). This increase in the C/N ratio under fully aerobic conditions would favour the heterotrophic growth of the facultative *Pseudomonas* spp. present on the reactor surfaces ($K_{NO2}^{Ps}$ = 0.31 mol. $m^{-3}$ and $K_{O2}^{Ps}$ = 2 *$10^{-3}$ mol. $m^{-3}$). The increase in the $CO_2$ - production rate indicates that indeed intensive oxidative reactions occurred in the reactor. The reasons and conditions under which heterotrophic denitrifying bacteria such as *Pseudomonas* spp. are able to produce nitrous oxide are not yet very clear. It appears that environmental stresses (e.g., pH or $O_2$ fluctuations, sub-optimal conditions) may be important (Grobben *et al*, 1994, Robertson & Kuenen, 1993). Though in some cases the $N_2O$ emissions tend to increase with decreasing availability of oxygen (Anderson and Levine, 1986, Grobben *et al*, 1994, Zumft, 1991), several

workers have also shown that NO and $N_2O$ production can be the result of aerobic denitrification by heterotrophic denitrifiers (Robertson & Kuenen 1989, 1990, Davies *et al,* 1989, Hochstein *et al.*1984). Therefore, we cannot draw definitive conclusions explaining the sudden nitrous oxide production, but this illustrates the need to carefully control and optimize the operation of such a system.

## CONCLUSIONS

A dynamic model has been developed to describe growth of nitrifying and denitrifying bacteria co-immobilized in double-layer gel beads. Reactor bulk concentrations and macroscopic consumption (production) rates were used to evaluate the model. The model predicted rather well the trend in the system's outputs, both in steady and dynamic conditions, although in absolute terms some discrepancies were observed. Simultaneous nitrification and denitrification at considerably high rates were achieved. Previously reported results on integrated nitrogen removal (Martins dos Santos, *et. al,* 1996a) were also well described by the model. Overestimations of the nitrification rates by the model seem to have been caused by diffusion limitation within the microcolonies as a result of low inoculum sizes. Denitrification rates higher than those predicted by the model were very likely caused by aerobic growth of *Pseudomonas* sp. attached to the reactor surfaces. Further quantitative evaluation of the model is thus still required. Therefore, we are presently investigating the system in more detail to be able to validate the model at particle level by determination of both the biomass and substrate (product) concentration profiles within the beads.

The model presented here has a mechanistic basis and is founded on general process equations and on the intrinsic kinetics of the cells. It has therefore potential applications in the modeling of other mixed cultures systems for integration of oxidative and reductive microbial processes such as in bioremediation and degradation of xenobiotics in waste streams.

## ACKNOWLEDGEMENTS

We thank Dr. Jan Hunik (Unilever Research Laboratories, The Netherlands) for his help and for very valuable discussions, and to Dr. Lesley Robertson (Delft University of Technology) for kindly providing the denitrifying cells and for helpful advises. A grant given by the Junta Nacional de Investigação Científica e Tecnológica (JNICT), Portugal is gratefully acknowledged as well.

## NOTATION

| | | |
|---|---|---|
| $D$ | dilution rate of the reactor | [$s^{-1}$] |
| $I$ | component | [-] |
| $m$ | microorganism | [-] |
| $\mathbf{D}_{gel}^{i}$ | diffusion coefficient component i in the gel | [$m^2.s^{-1}$] |
| $\mathbf{D}_{w}^{i}$ | diffusion coefficient component i in water | [$m^2.s^{-1}$] |
| $k_{l,g}^{i}$ | gas-liquid mass-transfer coefficient component i | [$m.s^{-1}$] |
| $k_{l,s}^{i}$ | liquid-solid mass-transfer coefficient component i | [$m.s^{-1}$] |
| $K_{i}^{m}$ | monod constant *m* for i | [$mol.m^{-3}$] |
| $m_{i}^{m}$ | maintenance *m* on i | [$mol.\ kg^{-1}\ s^{-1}$] |
| $R$ | radius to the centre of the bead | [m] |
| $R_{max}$ | maximum radius of the gel bead | [m] |
| $R_s$ | substrate consumption rate | [$mol.m^3.s^{-1}$] |
| $R_x$ | growth of the bacteria | [$kg.m^{-3}$.s-1] |
| $R_s^{sus}$ | substrate production by suspended bacteria | [$mol.m^{-3}.s^{-1}$] |
| $R_x^{sus}$ | production of suspended biomass | [$kg.m^{-3}.s^{-1}$] |
| $S$ | substrate concentration | [$mol.m^{-3}$] |
| $S'$ | limiting substrate concentration | [$mol.m^{-3}$] |
| $S_{bulk}$ | substrate concentration in the bulk phase | [$mol.m^{-3}$] |
| $X$ | concentration of biomass | [$kg.m^{-3}$] |
| $X_{bulk}^{m}$ | concentration of suspended biomass *(m)* | [$kg.m^{-3}$] |
| $X_{max}^{m}$ | max. biomass conc. in the gel microorganism (m) | [kg.m-3] |
| $Y_{i}^{m}$ | yield microorganinsm (m) on substrate (i) | [kg/mol i] |
| $\mu_{max}^{m,i}$ | maximum growth rate of (m) on (i) | [$s^{-1}$] |

## REFERENCES


Almeida, J.S., Reis, M.A.M., Carrondo, M.J.T. Competition between nitrate and nitrite reduction in denitrification by *Pseudomonas fluorescens. Biotech. & Bioeng.* 1995,46:476-484.

Anderson, I.C. & Levine, J.S. Relative rates of NO and $N_2O$ production by nitrifiers, denitrifiers and nitrate respirers. *Appl. Env. Microb.*, 1986.51:938-945.

Bader, F.G.. Analysis of double-substrate limited growth. *Biotech. & Bioeng.* 1978, 20:183-202.

Beefink H.H., van der Heijden R.T.J.M., Heijnen J.J. Maintenance requirements: energy supply from simultaneous respiration and substrate consumption. *FEMS Microbiology Ecology.* 1990 73:203-210

Beunink, H., Rehm, H.J. Synchronous anaerobic and aerobic degradation of DDT by an

immobilized mixed culture system. *Appl. Micro. Biotech.*1988,29:72-80.

Beunink, H., Rehm, H.J. Coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol by a co-immobilize mixed culture system. *Appl. Micro. Biotech.* 1990, 34:108-115.

Davies, K., Lloyd, D., Boddy, L. (1989). The effect of oxygen on denitrification in *Paracoccus denitrificans* and *Pseudomonas aeuroginosa*. *J. General Microbiology.* 1989, 135:2445-51

Fathepure, B.Z. Vogel, V.M. (1991). Complete degradation of polychlorinated hydrocarbons by a two-stage biofilm reactor. *Appl. Environ. Microbiol.* 1991.57(12):3418-3422.

Gardin H., Pauss A. Co-immobilization of aerobic and anaerobic bacterial communities in a mixed k-carrageenan/gelatin gel for the degradation of 2,4,6-thrichlorophenol, *Proc. International Workshop Bioencapsulation IV*, Quebec, Canada, 1994, pp 93-97

Gerritse, J. and Gottschal J.C. Mineralization of the herbicide 2,3,6-thrichlorobenzoic acid by a cocultureof aerobic and anaerobic bacteria. *FEMS Microbioly Ecology*, 1992, 10:89-98

Gooijer de C.D., Wijffels R.H., Tramper J. Growth and substrate consumption of Nitrobacter agilis cells immobilized in carrageenan: part 1. Dynamic modeling. *Biotechnology and Bioengineering*, 1991, 38:224-231.

Grobben, N. Robertson, L. & Kuenen, G. Nitrous oxide production by denitrifiers. *Book of Abstracts 5th Netherlands Congress on Biotechnology.* 1994, PJ-4.

Gujer W., M. Boller. A Mathematical model for rotating biological contactors. *Proc. EWPCA Conference on Technical advances in biofilm reactors,* Nice, France. 1989 pp. 69-89.

Hochstein, L.I., Betlach, M & Krtikos, G. The effect on denitrification during steady-state growth of *Paracoccus halodenitrificans. Archives Microbiology.* 1984, 137:74-78.

Hunik J.H. Tramper J., Meijer H.J.G. Kinetics of *Nitrosomonas europaea* at extreme substrate, product and salt concentrations. *Appl. Microb. and Biotechnol.*1992, 37:802-807.

Hunik J.H., Bos C.G., Van Den Hoogen P., De Gooijer C.D., Tramper J. Co-immobilized *Nitrosomonas euroapaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in k-carrageenan gel beads. *Biotechnol. Bioeng.* 1994, 43:1153-1163.

Koike I. & Hattori A. Growth yield of a denitrifying bacterium, *Pseudomonas Denitrificans*, under aerobic and denitrifying conditions. *Journal of General Microbiology,* 1975 88, 1-10.

Mankad, T. and Nauman, E.B. Modeling of microbial growth under dual limitations. *The Chemical Engineering Journal* 1992, 48:B9-B11

Martins dos Santos V.A.P.M. , Tramper J., Wijffels R.H. Simultaneous nitrification and denitrification using immobilized micro-organisms. *Biomat., Art. Cells & Immob. Biotech.*, 1993, 21(3),317-322.

Martins dos Santos V.A.P.M., Tramper J., Wijffels R.H. The Magic Bead concept: an integrated approach for nitrogen removal. (1996) Appl. Microbiol. Biotechnol. 45:447-

453.

Martins dos Santos, V.A.P., Vasilevska T., Kajuk, B., Tramper, J and R.H. Wijffels. (1997). Production and Characterization of Double-Layer Beads for Co-immobilization of Microbial Cells. *Biotechnology Annual Review*, 3: 227-244

Perry & Green. *Perry's Chemical Engineers Handbook,* 6th Ed. McGraw-Hill International, New York. 1984 pp. 3-258.

Press, W.H. *Numerical recipes in Pascal: the art of scientific computing.* 1989 Cambridge University Press, Cambridge, UK.

Robertson L.A., Corneline R., De Vos P., Hadioetomo R., Kuenen J.G. Aerobic denitrification in various heterotrophic nitrifiers. *Antonie van Leeuwenhoek*, 1989 56:289-299.

Robertson L.A. & Kuenen J.G. Combined heterotrophic nitrification and aerobic denitrification in *Thiosphera pantotropha* and other bacteria. *Antonie van Leeuwenhoek,* 1990, 57:139-152

Ryder D.N. and Sinclair, C.G. Model for the growth of aerobic micro-organisms under oxygen limiting conditions, *Biotechnology and Bioengineering*, 1972, 14:787-798.

Tramper, J. Nitrification and denitrification by immobilized bacteria, *Proc. 3rd European Congress on Biotechnology*, VCH,1984, Vol. IV :363-368.

Van Niel E.W.J. Nitrification by heterotrophic denitrifiers and its relation to autotrophic nitrification. *PhD dissertation*, 1991,chapter 4, pp 61-78.

Van Niel, E.W.J., P.A.M. Arts, B.J. Wesselink, L.A. Robertson and J.G. Kuenen. Competition between heterotrophic and autotrophic nitrifiers for ammonia in chemostat cultures. *FEMS Microbiology Ecology.*1993102: 109-118.

Van Ginkel C.G., Tramper J., Luyben K.Ch.A.M., Klapwijk A. Characterization of *Nitrosomonas europaea* immobilized in calcium alginate. *Enz. Microb. Technol.* , 1983, 5:297-303

Wang, J.H., Baltzis, B.C. G.A. Lewandowski. Fundamental denitrification studies with *Pseudomonas denitrificans*. *Biotech. & Bioeng.*, 1995, 47:26-41.

Wijffels R.H., De Gooijer C.D., Kortekaas S., Tramper J. Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in k-carrageenan. Part 2: Model evaluation. *Biotechnol. Bioeng.* 1991, 38: 232-240

Wijffels R.H., Schepers A.W., Smit M., de Gooijer C.D., Tramper J. Effect of initial biomass concentration on the growth of immobilized *Nitrosomonas Europaea*. *Appl. Microb. Biotech.* 1994, 42:153-157

Wijffels R.H., de Gooijer C.D., Schepers A.W., Beuling E.E., Mallée L.F., Tramper J. Dynamic Modeling of immobilized *Nitrosomonas Europaea*: implementation of diffusion limitation over expanding micro-colonies. *Enz. & MIcrob Tech.* 1995, 17:462-471

Willaert, R. Reaction and diffusion in gel-immobilized cell systems. PhD dissertation, 1993, Free University of Brussels, Belgium.

Zumft, W.G. *In*: The denitrifying prokaryotes. A.Balows, H. Truper, M Dworkin, W. Harder, and K. Schleifer (eds). *The prokaryotes*. 1991, p55-556. Springer-Verlag, New York

## APPENDIX

To determine the "most-limiting" substrate the Monod terms of the substrates involved are calculated. As discussed above, according to the non-interactive model (Mankad &Nauman, 1992) the specific growth rate of a micro-organism is limited by a single substrate at the time. The limiting substrate is that for which the Monod term is the smallest. The specific growth rate is therefore given by:

$$\mu = \mu_{max} min\left( \frac{S^1}{S^1 + K_{S^1}}, \frac{S^2}{S^2 + K_{S^2}} \right) \tag{1}$$

When the "most" limiting substrate ($S^i$) for a microorganism (m) has been determined, the rate ($r_S$) of substrate utilization is given by:

$$-r_s = \frac{dS}{dt} = \left( \frac{\mu^m{}_{max}}{Y_{m,s}} + m^m{}_s \right) \left( \frac{S^i}{S^i + K_{S^i}} \right) X^m \tag{2}$$

The biomass production rate for microorganism (m) and limiting substrate ($S^i$) is therefore defined as :

$$r_X{}^m = \frac{dX^m}{dt} = \mu^m{}_{max} \left( \frac{S^i}{S^i + K_{S^i}} \right) X^m - m_s{}^m X^m Y_s{}^m \left( 1 - \frac{S^i}{S^i + K_{S^i}} \right) \tag{3}$$

Applying a differential mass balance over a biocatalyst bead for simultaneous diffusion and consumption of substrate we come at:

$$\frac{\partial S^i}{\partial t} = D^i{}_{gel} \left( \frac{\partial^2 S^i}{\partial r^2} + \frac{2}{r} \frac{\partial S^i}{\partial r} \right) - r_s \tag{4}$$

with boundary conditions:

$$\text{for r} = 0 \qquad D^i{}_{gel} \frac{\partial S^i}{\partial r} = 0$$

$$\text{for r} = \text{R} \qquad D^i{}_{gel} \frac{\partial S^i}{\partial r} = k^i{}_{l,s} \left( S^i{}_{bulk} - S^i{}_{surf} \right)$$

The model is based on the assumption of an ideally mixed reactor thus $S_{out}$ is equal to $S_{bulk}$. The corresponding mass balances over the reactor are thus given by:

$$\frac{dX^{m}{}_{bulk}}{dt} = D\left(X^{i}{}_{in_{in}} - X^{i}{}_{bulk}\right) + r^{susp}_{X^{m}} \tag{5}$$

and

$$\frac{dS^{i}_{bulk}}{dt} = D(S^{i}_{in} - S^{i}_{bulk}) - r^{susp}_{s} - k^{i}_{l,s}A\left(S^{i}_{bulk} - S^{i}_{surf}\right) \tag{6}$$

Eqs. 5 and 6 include terms corresponding to substrate consumption by suspended biomass ($r_S{}^{susp}$) and bacterial growth in the bulk ($r_X{}^{susp}$) as a result of released cells.

This chapter has been submitted for publication by Martins dos Santos VAP, Verschuren PG, Van den Heuvel JC, Tramper J, Wijffels RH.

# CHAPTER 8

## Substrate and Product Profiles Across Double-Layered (De)Nitrifying Biocatalysts: Dynamic Modelling and Validation


## ABSTRACT

A previously developed dynamic model describing integrated nitrification and denitrification by nitrifying and denitrifying bacteria co-immobilized in the separate layers of double-layer gel beads was validated under a wide range of conditions. The $O_2$, $NH_4^+$ and $NO_2^-$ gradients across a double-layered slab with co-immobilized *Nitrosomonas europaea* and *Pseudomonas* spp. were measured regularly with microelectrodes and compared to model simulations under nitrifying, denitrifying and coupled nitrifying and denitrifying conditions. The model describes diffusion of components, substrate utilization and growth, all occurring simultaneously within the biocatalytic support The model predicts biomass and solute bulk concentrations, substrate consumption rates, product formation rates, and biomass and solute concentration profiles within the beads as function of time. Fluctuations in substrate load, dilution rates or mass transfer parameters are handled as well. Under all circumstances (i.e. denitrification only, nitrification only and coupled nitrification and denitrification), the predicted $O_2$, $NH_4^+$ and $NO_2^-$ microprofiles matched well the gradients measured experimentally. Also, the transitions from one set of conditions to the other were in all cases properly accounted for by the model. Moreover, the model described correctly the simultaneous use of oxygen and nitrite as electron acceptor by the heterotrophic denitrifying bacteria. However, likely due to its unstructured nature, it could handle only up to a limited extent the growth and activity of these cells on internal storage compounds such as polyhydroxyalkanoates. Nevertheless, this set of results taken altogether demonstrate that the model describes appropriately the behavior of these processes under both steady and dynamic conditions and that it is able to account for several subsidiary phenomena such as heterotrophic oxygen consumption. This is the first report on a detailed study of a coupled oxidative-reductive processes by co-immobilized bacteria that is supported by both a sound theoretical framework and an accurate experimental technique to describe these processes at biocatalyst level. Such a mechanistic model is thus not only a valuable tool for the design and development of each of the N-removal processes outlined above and under given steady conditions but also for the more general case of strongly changing environments, which is the most frequent in wastewater treatment practices.

## INTRODUCTION

Many of the modern wastewater treatment plants rely on the use of immobilized microorganisms (either artificially entrapped into matrices or naturally attached onto carriers) for compact and effective removal of both inorganic and organic compounds. Unlike in well-mixed suspended-cell processes (such as activated-sludge basins), in immobilized-cell processes (biofilters, fluidized beds, gas-lift reactors, among others) cell growth and sequential elimination of pollutants may take place independently of the wastewater flow because the intervening microorganisms are retained in the treatment unit. These characteristics allow much more compactness and treatment effectiveness. Nevertheless, owing to additional mass-transfer resistance, pronounced gradients of substrates, products and, consequently, of biomass throughout the biocatalytic supports tend to develop in most immobilized-cell systems. In many aerobic treatment processes, for instance, these gradients may ultimately result in that up to 95% of the biocatalytic support remains unused (e.g. entrapped systems) because the relevant microorganisms grow in a rather thin layer (typically 100 to 200 μm thick) just underneath (or just above) the carrier surface (Kurosawa & Tanaka, 1990, Bryers & Banks, 1990, Wijffels *et al.*, 1995, Martins dos Santos *et al.*, 1996a).

However, despite its enormous relevance for the development and design of effective treatment systems, detailed monitoring and assessment of the changes of the underlying communities and metabolic activities within biofilms (both artificial and natural) is generally troublesome and inaccurate. Yet, during the last decade, many important advances in both microsensor technologies (Damgaard *et al.*, 1995, Santegoeds *et al*, 1998, Lens *et al.*, 1995) and techniques for *in-situ* identification and quantification of microbial cells (Amann, 1997, 1998, Lee *et al*, 1999) has made it possible to monitor accurately substrate and product gradients as well as metabolic activities at micrometer scale and to determine the distribution, structure and function of immobilized-cell communities in several treatment processes (de Beer *et al*, 1988, 1993, 1997, Dalsgaard *et al.*, 1995, Sweerts *et al.*, 1989,1990, Ramsing *et al.*, 1993, Schramm *et al*, 1996, 1999a,b, Santegoeds *et al.* 1999, 1998, Okabe et. 1996, 1999a,b, Lee *et al*, 1999, Zhang and Bishop, 1995, 1996).

### *Process dynamics*

Notwithstanding the fact that much and very valuable information on the local bacterial activities, actual reactant fluxes, biofilm structure and microbial interactions has been obtained through the above-mentioned studies, they all lacked a mechanistic theoretical framework able to account for the complex interplay of the underlying processes. Indeed, although several of the above-mentioned research reports used empirical models

to fit the concentration gradients or fluxes obtained, their predictive value for conditions other than those under which they were measured (and namely under dynamic conditions) is very limited and of little relevance for the quantitative design and optimization of effective systems based on such immobilized-cell technologies.

Hence, aiming at establishing a sound mechanistic framework for describing simultaneous nitrification and denitrification by co-immobilized bacteria, we have recently developed a dynamic mathematical model that incorporates simultaneous mass transfer, substrate consumption and growth (Martins dos Santos *et al.*, 1996b, *2001*). The model has been applied to an engineered system that couples nitrification and denitrification in a compact, single reactor in which pure nitrifying and denitrifying cells are co-immobilized in double-layered gel beads. This model has been successfully evaluated both under steady and dynamic conditions and at reactor level by comparing the effluent concentrations of substrates and products with predicted values (Martins dos Santos *et al.*, 1996b).

The macroscopic behavior of such a system, however, is intrinsically determined by the interplay of the several complex processes (mass transfer, substrate consumption, growth, inhibition and so forth) occurring simultaneously within each individual biocatalyst particle. A thorough validation of a model aiming at describing such processes as well as their dynamics requires thus measuring local (and time-dependent) concentrations of substrates, intermediates and products for a given range of relevant operating conditions. Hence, in this paper, the model is validated by comparing the predicted substrate and product profiles throughout the biocatalysts with those measured with ion-selective and oxygen microelectrodes under different operating conditions. This study aimed at gaining more insight and understanding into the complex mechanisms underlying these processes so that this knowledge can be used for the design, optimization and scale up of compact nitrogen removal systems.

## MATERIALS AND METHODS

### Strains and immobilization procedure

*Nitrosomonas europaea* ATCC 19718 and *Pseudomonas* spp., strain LMD 84.60 (formerly *Pseudomonas denitrificans*) were cultivated, harvested, concentrated and prepared for immobilization as described before (Martins dos Santos *et al.*, 1996a,b). The concentrated nitrifying and denitrifying cell suspensions were separately mixed with a known amount of an agar solution at 39°C so to reach a final agar concentration of 20 $g.dm^{-3}$. Part of the denitrifying gel suspension was then poured into a cylindrical vessel (diameter, 11.5 cm; height, 6 cm) containing four equally-spaced square baffles (1cm). After cooling, a solid gel slab (5 mm thick) was formed on the bottom of the

vessel. Part of the nitrifying gel suspension was then poured onto this slab to form a 2-mm thick agar layer containing the nitrifying cells over the 5-mm denitrifying layer after cooling. The remaining of both gel suspensions was used to produce nitrifying and denitrifying gel beads (in a similar way as described by Hellendoorn *et al*, 1998), which were subsequently used to measure the initial nitrifying and denitrifying cell concentrations.

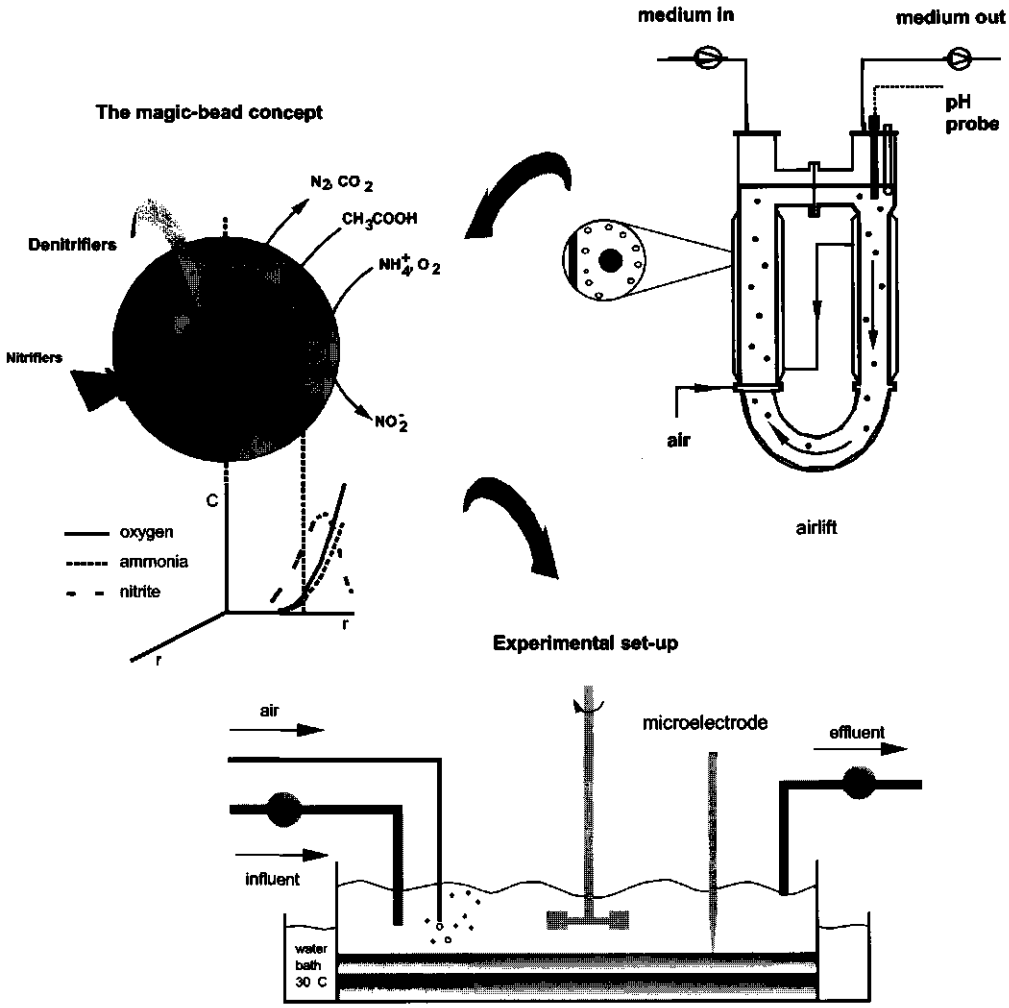


Figure 1 - Schematic representation of the Magic-Bead concept and the experimental set up.

**Immobilized-cell cultivation**

The vessel containing the double-layered agar slab was placed in a Faraday cage and was used as a continuous stirred submerged reactor in a similar way as that described by Wijffels *et al.* (1995) (see also Figure 1).

The reactor medium (Martins dos Santos *et al.*, 1996b), pH 7.3, supplied at a dilution rate of $5.56*10^{-4}$ $s^{-1}$, contained 0.4 mM $NH_4^+$ and, whenever desired, was supplemented with 0.5 mM acetate and 0.2 mM nitrite to provide denitrifying conditions. Oxygen, ammonium and nitrite profiles across both layers of the plate were measured at different time steps under denitrifying, nitrifying and simultaneous nitrifying/denitrifying conditions. Air was supplied to the reactor through a small sparger at a rate of about 10 $ml.s^{-1}$. The reactor was maintained at 30°C and was stirred by an air-driven six-blade stirrer placed at 2 cm above the gel surface.

**Immobilized-cell concentration**

The initial biomass concentration in the gel layers was determined through activity assays of the agar beads containing either nitrifiers or denitrifiers in a biological oxygen monitor as described before (Martins dos Santos, 1996b). To estimate the lethality of the immobilization procedure, these concentrations (dilution-corrected) were compared to those of the individual concentrated cell suspensions that were also measured by activity measurements.

**Ion-selective and oxygen microelectrodes**

All LIX components, cyclohexanone, and N,N-dimethyltrimethylsilylamine were obtained from Fluka (selectophore quality).

Single-barelled boro-silicate glass capillaries (Ø=1.2 mm, GC 120F-15, Clark Electromedical Instruments, Pangbourne, Reading, U.K.) were drawn manually to microcapillaries with diameters of 8-10 μm for nitrite electrodes. The tips were silanised with N,N-dimethyltrimethylsilylamine for 16 hours at 150°C in a similar vessel as described previously (Tsien and Rink, 1980). The micropipettes were back-filled with electrolyte, 10 mM $NaNO_2$, 10 mM NaCl, and 50 mM sodiumphosphate buffer adjusted to pH 7.0. The tips were then briefly dipped in the liquid membrane phase (diluted twice their weight with cyclohexanone) and allowed to dry for 4 hours, resulting in membranes of 100-300 μm thick after drying. Nitrite membranes consisted of 10 % (w/w) nitrite Ionophore I, 2 % (w/w) potassium tetrakis(4-chlorophenyl)borate (KTpClPB), and 10 % (w/w) PVC in o-NPOE and showed in the medium applied a log-linear response down to $10^{-5}$ $mol \cdot m^{-3}$ with a slope of -56 mV/decade. ). A minimal tip diameter of 8 μm was necessary to obtain a similar response as the macroelectrode

described by Schaller *et al.* (1994). Non-shielded ammonium electrodes with a tip diameter of 2-3 μm were prepared as previously described (De Beer *et al.*, 1997), and had a log-linear response of $10^{-4}$ mol·m$^{-3}$, and a slope of 56mV/decade.Finally, the electrodes were mounted in a connecting holder containing 0.3 M KCl and a chlorinated silver wire. The electrode assembly, mounted on a micromanipulator was placed inside the Faraday cage to reduce electromagnetic interference. The fluid-flow system was electrically separated from the outside by trickling devices. The electrical potential (EMF) was measured against a reference electrode of the Ag/AgCl type (Crytur, RAE 113) with a shielded cable connected to a high impedance electrometer (Keithley 617) outside the cage connected to a personal computer. Both ion-selective microelectrodes were calibrated in dilution series of either nitrite or ammonium directly in the reactor using the same medium and under the same conditions (temperature, stirring and flow) as those for the (de)nitrifying experiments.

Combined oxygen microelectrodes with internal references were prepared according to Revsbech *et al.* (1980). These microlectrodes had a tip diameter of 8 μm and were barely sensitive to stirring (<3% change in signal). Both the ion-selective and oxygen microelectrodes were mounted on a motor-driven micromanipulator (OWIS) allowing a spatial resolution of 1 μm.

**Other analytical methods**

Ammonia, nitrite and nitrate (as a control) concentrations in the influent and effluent of the continuous reactors were measured using a continuous-flow analysis system (Autoanalyser SB40, Skalar Methods) as earlier described (Hunik, 1994). Influent and effluent acetate concentrations were measured by gas chromatography as described before (Martins dos Santos *et al*, 1996b).

**Measurement of the effective diffusion coefficients ($D_{eff}$)**

The effective diffusion coefficients for the nitrite and ammonium ions in 2% (w/v) agar slabs were measured through a diffusion cell (Axelsson *et al*, 1991) by applying the pseudo-steady-state method described by Beuling (1996) and Westrin (1991). The reliable use of this methodology requires the resistance to external mass transfer to be negligible (Westrin, 1991). This can be considered so if the Biot number ($Bi = (k_{l,s} L) / D_{s,gel}$) approaches infinity or, at least, if it is sufficiently larger than one (Bird *et al.*, 1960). Although the diffusion-cell consisted of a circular membrane fixed in space separating two fluids stirred at constant speed, for estimation of the Biot number we considered the membrane as being a planar disk immersed in a fluid and rotating at

constant speed. For these conditions, the following correlation was used to estimate the external mass transfer coefficient $k_{l,s}$ (Sherwood *et al*, 1975):

$$k_{l,s} = 0.62\ D_s^{\ 2/3}\ \nu^{-1/6}\ \omega^{1/2} \qquad (1)$$

where $D_s$ is the solute diffusion coefficient in water at 303 K and infinite dilution ($2.19*10^{-9}$ $m^2.s^{-1}$ for $NO_2^-$ and $2.23*10^{-9}$ $m^2.s^{-1}$ for $NH_4^+$), $\nu$ is the kinematic viscosity of the fluid at 303 K ($0.857*10^{-6}$ $m^2.s^{-1}$ for water) and $\omega$ the angular velocity in rad $s^{-1}$ (calculated as $2*\pi*rps$). Using these relations and estimating an approximate diffusion coefficient in gel of $2*10^{-9}$ (about 90% of that in water) we came at a Bi number of 122, which we considered high enough so to neglect external mass diffusion for measurement purposes.

**Measurement of the liquid-solid mass-transfer coefficients ($k_{l,s}$)**

Before determining the relevant mass-transfer coefficients, it was checked whether there was any variation in the thickness of the Diffusive Boundary Layer (DBL) as a function of the distance from the stirrer. To this end, several oxygen microprofiles were measured at the borders of a 2.5 cm concentric agar strip. As these profiles were virtually equal, we concluded that the hydrodynamic conditions above this circular strip were the same. Therefore, all microelectrode measurements were made within this strip.

*Measurement of the thickness ($\delta$) of the Diffusive Boundary Layer (DBL)*

The thickness of the DBL (hypothetical layer through which solute diffusion takes place and where most of the mass-transfer resistance is located) was determined from oxygen profiles measured across the liquid-solid interface by applying a non-linear regression to all data points as done before (Wijffels *et al*, 1995, de Beer *et al.*, 1993). The only difference with regard to these earlier studies was that the exact location of the agar surface (x=0) was determined with more accuracy by emptying the vessel and by recording the signal of the oxygen electrode as it moved (in steps of 1-5 μm) from the neighborhood of the surface down into the gel. The reasoning behind this was that the signal of an oxygen electrode changes abruptly if it comes from a gas phase and touches a solid surface. To confirm the position of this interface, the second derivatives of the non-linear fits of complete profiles were calculated and the inflexion point was accurately determined. The combination of these methods allowed an accuracy of ± 4 μm in determining the location of the gel surface.

The liquid-solid mass-transfer coefficient for solute i ($K_{l,s}^i$) was then calculated by the film theory as $D_w^i / \delta$ (Bird *et al.*, 1960).

**Experimental strategy**

Initially, the flow-through reactor with *Ns. europaea* and *Pseudomonas* sp. co-immobilized in the double-layered slab was subjected to predominantly denitrifying conditions. In practice this was done by sparging nitrogen instead of air at the same flow rate (10 $ml.min^{-1}$) as in the measurement of mass-transfer coefficients to avoid any change in the hydrodynamic conditions (see above). $NO_2^-$ and acetate were added to the medium at a final concentration of 0.2 and 0.5 mM, respectively. Ammonium at 0.4 mM was supplied as well to account for residual nitrification. In the model these predominantly denitrifying conditions were accounted for by setting a bulk oxygen concentration at a value 10 times lower than that of its saturation in water. At day 6 after start-up, the acetate supply was interrupted and nitrogen was substituted by air. The system was thereafter run under purely nitrifying conditions. Six days later acetate was again continuously added to the influent medium to a final concentration of 0.5 mM so that coupled nitrification and denitrification could take place.

To study the dynamics of nitrification and coupled nitrification and denitrification a similar experiment was set up and the corresponding ammonium, nitirite and oxygen profiles were measured along time.

**Dynamic model**

The metabolically unstructured model, developed to describe the dynamic behavior of the system represented in Figure 1, has been earlier presented and evaluated at macroscopic (reactor) level (Martins dos Santos *et al.*, 1996b). In the present paper, the model is thoroughly validated by measuring substrate and product concentrations across the biocatalyst for different operating conditions.

For the experimental set up shown in Figure 1, the equations describing diffusion in the original model for a sphere were modified for a "double" (infinite) flat plate. The set of (partial) differential equations was solved numerically using a fourth-order Runge-Kutta algorithm. The model predicts substrate and biomass concentration profiles across the slab as a function of time and from that the overall substrate consumption and product formation rates. Fluctuations of dilution rates, substrate loading and mass transfer parameters in time are handled by the model as well. External mass transfer between the bulk and the slab is accounted for (Martins dos Santos *et al.*, 1996). The model parameters are shown in Table I and were those used in Martins dos Santos *et al.* (1996b) except for the effective diffusion coefficients for $NO_2^-$ and $NH_4^+$ in agar and for the mass-transfer coefficients between fluid and agar that were measured experimentally as above described. The diffusion coefficient of oxygen in agar was that measured by Hulst *et al.* (1989).

**Table 1 – Model parameters**

| Parameter | Value | Dimensions | Parameter | Value | Dimensions |
|---|---|---|---|---|---|
| $\mu_{max,O2}^{Ps}$ | $2.78 \times 10^{-5}$ | $s^{-1}$ | $X^{Ps}$ | 170 | $kg.m^{-3}$ |
| $\mu_{max,NO2}^{Ps}$ | $3.33 \times 10^{-5}$ | $s^{-1}$ | $K_{l,s}^{NH4}$ | $2.21 \times 10^{-5}$ | $m.s^{-1}$ |
| $\mu_{max}^{Ns}$ | $1.52 \times 10^{-5}$ | $s^{-1}$ | $K_{l,s}^{NO2}$ | $2.21 \times 10^{-5}$ | $m.s^{-1}$ |
| $m_{O2}^{Ns}$ | $9.4 \times 10^{-4}$ | mol $O_2.kg^{-1}.s^{-1}$ | $K_{l,s}^{O2}$ | $2.63 \times 10^{-5}$ | $m.s^{-1}$ |
| $m_{NO2}^{Ps}$ | $5.44 \times 10^{-4}$ | mol $NO_2.kg^{-1}.s^{-1}$ | $K_{l,s}^{N2}$ | $2.63 \times 10^{-5}$ | $m.s^{-1}$ |
| $m_{O2}^{Ps}$ | $3.84 \times 10^{-4}$ | mol HAc.$kg^{-1}.s^{-1}$ | $K_{l,s}^{HAC}$ | $2.07 \times 10^{-5}$ | $m.s^{-1}$ |
| $Y_{NH4}^{Ns}$ | $1,66 \times 10^{-3}$ | $kg.mol^{-1}$ $NH_4$ | $D_w^{NH4}$ | $2.20 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{O2}^{Ns}$ | $1.19 \times 10^{-3}$ | $kg.mol^{-1}$ $O_2$ | $D_w^{NO2}$ | $2.20 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{O2}^{Ps}$ | $9.86 \times 10^{-3}$ | $kg.mol^{-1}$ $O_2$ | $D_w^{N2}$ | $2.83 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{NO2}^{Ps}$ | $7.39 \times 10^{-3}$ | $kg.mol^{-1}$ $NO_2$ | $D_w^{O2}$ | $2.83 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{HAC}^{Ps}$ | $1.37 \times 10^{-2}$ | $kg.mol^{-1}$ HAc* | $D_w^{HAC}$ | $2.00 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{O2}^{Ns}$ | $5.05 \times 10^{-3}$ | $mol.m^{-3}$ | $D_g^{NH4}$ | $1.78 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{NH4}^{Ns}$ | 1,25 | $mol.m^{-3}$ | $D_g^{NO2}$ | $1.78 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{O2}^{Ps}$ | $2 \times 10^{-3}$ | $mol.m^{-3}$ | $D_g^{N2}$ | $2.30 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{NO2}^{Ps}$ | 0,31 | $mol.m^{-3}$ | $D_g^{O2}$ | $2.30 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{HAC}^{Ps}$ | 0,73 | $mol.m^{-3}$ | $D_g^{HAC}$ | $1.62 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $X^{Ps}$ | 36,7 | $kg.m^{-3}$ | | * HAc - Acetate | |

## RESULTS AND DISCUSSION

### Model parameters

#### *Effective diffusion coefficients*

Following the calculation procedure described by Beuling *et al.* (1996), and Westrin *et al.* (1991) for a pseudo-steady-state diffusion-cell (assuming Fickian diffusion), we arrived at an effective diffusion coefficient $D_{eff}$ of $1.96 \pm 0.06 * 10^{-9}$ $m^2s^{-1}$ for $NO_2^-$ and $2.04 \pm 0.05 * 10^{-9}$ $m^2s^{-1}$ for $NH_4^+$. This represents about 0.90 of the diffusion coefficient ($D_0$) of these ions in water at infinite dilution at 303 K and viscosity of 0.795 mPa.s through the Stokes-Einstein equation (Lide, 1992). Such values agree well with those obtained using the equation $D_{eff} = D_0 (1 - \Phi_p)^3 / (1 + \Phi_p)^2$ of Makie and Meares (1955), in which $\Phi_p$ represents the volume fraction of gel that is inaccessible to the diffusant due to the presence of polymer (here assumed to equal the volume fraction of agar, 2% (v/v)). This empirical relation implicitly assumes "a gel with a fine polymer structure with a chain thickness comparable to the molecular jump distance" in which only physical hindrance of the ions is considered (Westrin, 1991). This approach seems valid for this range of concentrations because the calculated values for the activity coefficients are close to unity.

***Liquid-solid mass transfer coefficients***

Although in many studies the influence of the external diffusion limitation on the performance of immobilized-cell systems has been neglected (see Wijffels and Tramper, 1995 for an overview), other detailed investigations have shown that this parameter often determines both their kinetic behavior and the maximum attainable reaction levels (De Gooijer *et al*, 1991, Wijffels *et al*, 1991, 1995, Hooijmans *et al*, 1990a). Indeed, a sensitivity analysis of the model here described shows that the transfer of oxygen from the liquid to the solid phase is, under a wide range of operating conditions, the rate-limiting step in the overall conversion of ammonium to nitrogen gas (Martins dos Santos *et al.*, 1996b).

*Measurement of the thickness of the Diffusive Boundary Layer (DBL)*

It is clear from Figure 2 that the stirring rate influenced the thickness of the boundary layer. However, this influence diminished strongly for higher rates, for which a difference lower than 8 μm in the thickness of the DBL was found.

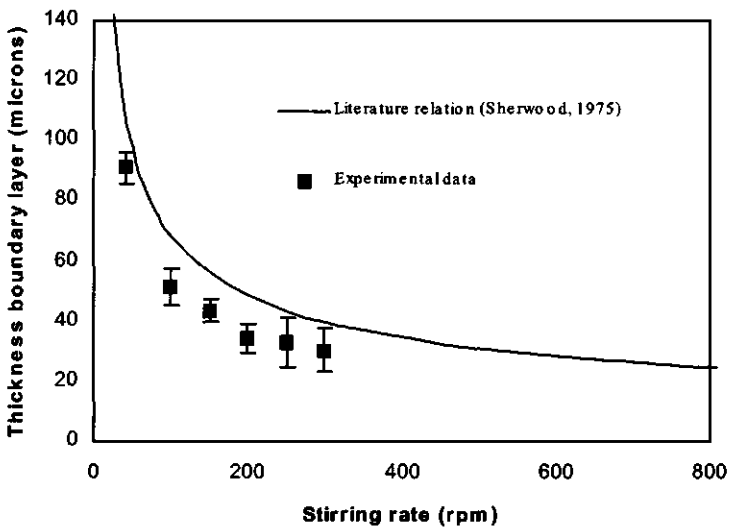


Figure 2 – Experimental (points) and estimated (solid line) thickness of the boundary layer as a function of the stirring rate. Error bars for standard deviation refer to an average of four measurements.

Hence, all subsequent measurements were carried out at a stirring rate of 150 rpm [at rates higher than 200 rpm, the turbulence affected negatively the measurements with ion-selective microelectrodes and caused vibration of the gel slab]. The thickness of the

boundary layers calculated at this rate was similar to those obtained by Wijffels *et al.* (1995) for a similar set up, but were considerably lower than those found around gel beads in other microelectrode studies (Cronenberg *et al.* 1991, Hooijmans *et al.* 1990b, de Beer *et al* 1993). This is likely due to the different flow regimes applied and/or the different geometry of the biocatalytic support.

It is also noteworthy that estimations from an empirical relation (based on a rapidly rotating disk immersed in a fluid as defined by Sherwood, 1975, see also Eq.(1)) gave thicknesses consistently higher than those measured with the microelectrodes (points in Figure 2). The tendency is, however, quite similar. This discrepancy between estimated and measured values is likely due to both the fact that the empirical relation is in fact a rough simplification of reality (gel slab as a rapidly rotating disk immersed in a fluid) and, perhaps more important, due to the extra turbulence introduced through aeration.

**(De)nitrification profiles**

Initially, the reactor was subjected to predominantly denitrifying conditions for about six days. Thereafter, the acetate supply was interrupted, and the system was operated under purely nitrifying conditions. Six days after the supply was stopped acetate was again added to the medium to and the reactor was operated further under coupled nitrifying and denitrifying conditions. The substrate and product gradients across the slab measured for day 5, 10 and 12 of the experiment are shown in Figures 3 and 4.

***Denitrification***

Figure 3 shows the predicted and measured substrate and product concentration profiles for the system under such conditions at day 5 after start up.

Clearly, model predictions and measurements matched well, especially those regarding nitrite and ammonium. Due to the low oxygen concentration, there was only a little nitrification within the very first outer layers, as becomes clear from the almost flat ammonium profile. Nitrite, on the contrary, was consumed at a considerable rate within the first layers of the denitrifying zone. The shape of the nitrite profile reflects its diffusion across the nitrifying zone, being driven by the concentration gradient between the bulk and the denitrifying layer, which virtually works as a "sink". From the Figure it seems also clear that the *Pseudomonas* spp. within the first layers of the denitrifying zone would have accounted for most of the denitrification.

The actual oxygen profile was sharper than predicted likely due to its uptake at the slab surface by undefined heterotrophic populations. In fact, under these conditions (retention time of 30 min, intensive mixing and abundant supply of acetate) heterotrophic microorganisms tend to attach to surfaces and form biofilms as survival

strategy (Heijnen *et al.*1992, Tijhuis *et al.*, 1994, Martins dos Santos *et al.*, 1996b, Van Benthum *et al*, 1997). Such a biofilm (quite thin, though) could be observed some days after start-up.

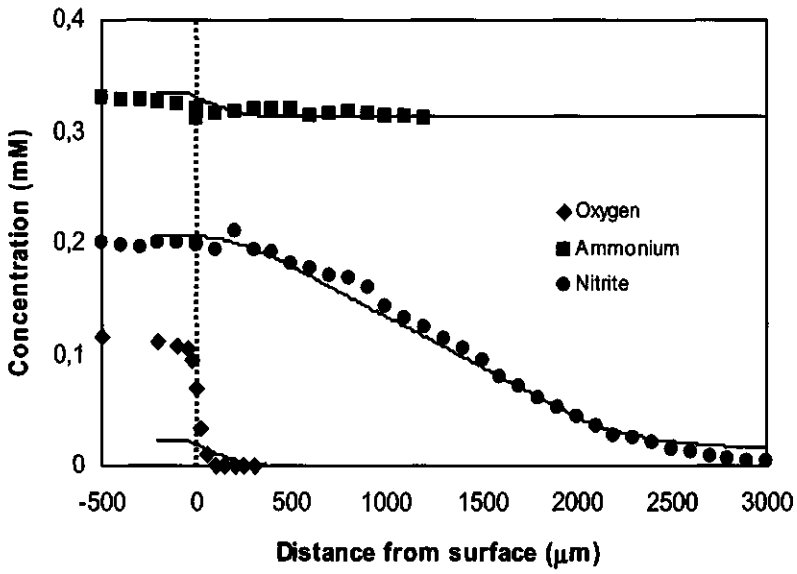


Figure 3 – Predicted (solid lines) and measured (symbols) substrate profiles across the double-layer gel slab for denitrifying conditions. The vertical line indicates begin of denitrifying zone. Zero represents the slab surface. From left to right: bulk liquid: -500 to –52 μm; diffusive boundary layer: -52 to 0 μm; nitrifying layer: 0-2000 μm; denitrifying layer: beyond 2000 μm.

The oxygen uptake by this biofilm at the surface counterbalanced the relatively high oxygen tension in the bulk as oxygen was fully depleted at a depth of about 50μm. This oxygen level in the bulk (about 0.1 mM) was higher than predicted because it was difficult to maintain a constant (low) oxygen tension in such an open vessel. In any case, these discrepancies did not affect the denitrification process as a whole.

***Nitrification and coupled nitrification / denitrification***

Figure 4 shows a typical set of predicted and measured oxygen, ammonium and nitrite profiles across the double-layered gel slab under nitrifying (4A) and coupled nitrifying and denitrifying (4B) conditions (i.e., in the absence and the presence of an organic C-source, respectively). To increase accuracy and minimize errors we chose bulk N-limiting conditions so that the profiles could be measured throughout a large part of the biocatalytic support for relatively long periods of time.

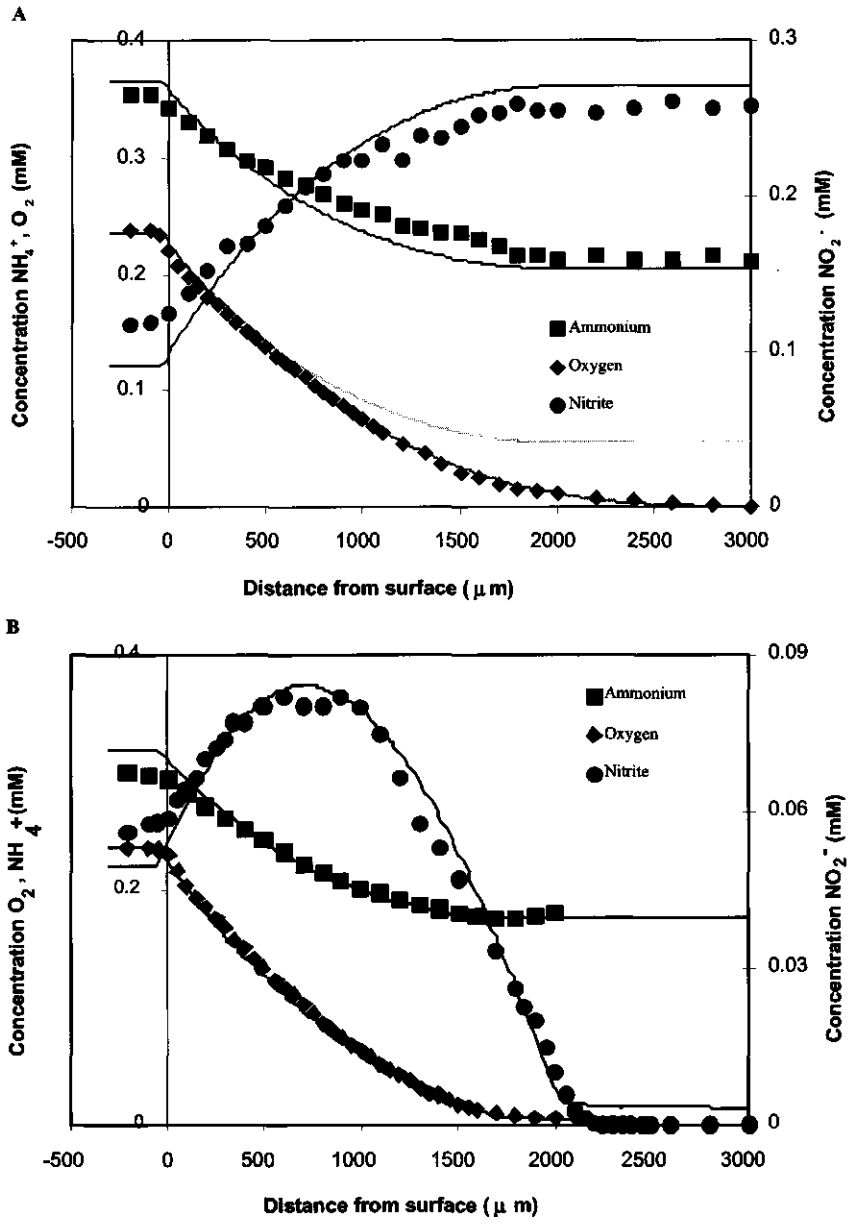


Figure 4 – Predicted (black lines) and measured (symbols) substrate and product profiles across the double-layer slab under nitrifying (A) and simultaneous nitrifying and denitrifying conditions (B). Grey line in (A) represents the model predictions for purely nitrifying conditions. Note that, to facilitate visual analysis, different scales are used for nitrite in A and B.

Clearly, and except for some slight deviations, in both situations the predicted and measured profiles agreed well with each other. This agreement remained so throughout a series of repetitive experiments under several different conditions.

Figure 4A shows that ammonium is consumed at about the stoichiometric rate of nitrite accumulation. All ammonium that is consumed is thus transformed into nitrite. The shape of the curves indicates that nitrifiers are active throughout the whole 2000-μm layer (4A) and that nitrite is not further converted into nitrogen through denitrification. Despite the generally good agreement between model predictions and experimental results in Figure 4A, it is important to point out that the oxygen gradient predicted by the model under purely nitrifying conditions (grey line in 4A) clearly underestimated oxygen consumption in the flattened part of the profile, in which about 0.05 mM oxygen would have remained unused. As it is discussed below, this deviation was likely due to heterotrophic oxygen uptake by the denitrifying bacteria in the inner slab. By taking into account this contribution (by setting a residual concentration of acetate [0.05mM] predictions (black line) and experimental data (diamonds) matched rather well.

In 4B, ammonium, oxygen and nitrite profiles were measured under simultaneously nitrifying and denitrifying conditions. Likewise 4A, model predictions and experimental values matched well. [Note that, as in 4A, the predictions for oxygen took into account heterotrophic oxygen consumption]. The shape and magnitude of the ammonium and oxygen profiles are similar to those in 4A (only a little lower as a result of two extra days of nitrifying activity) but that of nitrite shows now a broad peak with a maximum at about 600 μm inside the slab. Also, its peak concentration is lower than that in 4A. These features in the nitrite profile of 4B reflect the simultaneous production of nitrite by *Nitrosomonas* within the nitrifying layer, and its consumption by *Pseudomonas* in the denitrifying one (beyond 2000 μm, see Figure 6 below). This sequential process for nitrfication and denitrification was hence proceeding simultaneously within the slab and at rates and magnitudes that were appropriately predicted by the model.

***Rate-limiting substrates***

The detailed analysis of Figure 4 (both A and B) showed that ammonium and not oxygen was actually limiting nitrification. The "Monod" term [$(S_i / (S_i + Ks_i)$ in which $S_i$ is the local concentration measured for compound i and $Ks_i$ the respective affinity constant by the cell), see Mankad & Nauman, 1992] for $NH_4^+$ is much lower than that for oxygen throughout the whole nitrifying layer. The "Monod" term gives an indication of what the cell "sees" and of what it is able to take up. For instance at 1500 μm, a

depth at which flattening is almost complete, the oxygen concentration of 0.035 mM is still about 7 times higher than 0.005 mM, which is the value for the affinity constant of *Nitrosomonas* for $O_2$. On the contrary, at this depth the local ammonium concentration (0.24 mM) is only one fifth of *Nitrosomonas*' calculated affinity constant (1.25 mM).
Hence, regardless whether an interactive (growth and consumption are dependent on the product of the Monod terms for oxygen and ammonium) or a non-interactive (growth and consumption dependent on the substrate for which the Monod term is the lowest) approach is used for analyzing such results (a controversial dichotomy widely discussed in the relevant literature), ammonium remains the substrate that determines the most, under these conditions, the performance and effectiveness of nitrification. In fact, the availability of ammonium determined not only the nitrification level but also the overall nitrogen removal rate as a whole since the rate of denitrification depended directly on the nitrite produced within the nitrifying layer. This became clear as well from the analysis of nitrogen, carbon and oxygen fluxes for coupled nitrification and denitrification in Figure 4B (not shown). Here too, the Monod term for ammonium was much smaller than that of oxygen for *Nitrosomonas*. Similarly, the Monod term for nitrite was lower than that for acetate throughout the whole denitrifying layer.
This brief analysis illustrates the importance of having a detailed insight into the mechanisms underlying the processes involved at biocatalyst level for the understanding and, consequently, for the improvement of a complex treatment system as a whole (such as coupled nitrification and denitrification).

**Dynamics**

For study of sequential nitrification and coupled nitrification and denitrification a separate, similar experiment was set up and the relevant solute profiles monitored in time. Initially the flow-through reactor with *Ns. europaea* and *Pseudomonas* sp. co-immobilized in the double-layered slab was subjected to purely nitrifying conditions (i.e., no organic C-source, full aeration and $NH_4^+$ 0.4 mM). Five days after start-up, acetate to a final concentration of 0.5 mM was added to the in-flowing medium, allowing thereby full heterotrophic denitrification to take place. At day 24, air sparging substituted nitrogen and nitrite was added at a final concentration of 0.16 mM. The outcome of the experiment is shown in Figures 5 and 6. Figure 5 shows the evolution in time of the ammonium (5A), oxygen (5B) and nitrite (5C) profiles across the doubled-layered slab throughout the whole experiment. In the first few days after start up, the profiles (in particular those of ammonium and oxygen) were relatively flat. As growth proceeded, however, the gradients increased and became progressively steeper towards the last days of the measurements.

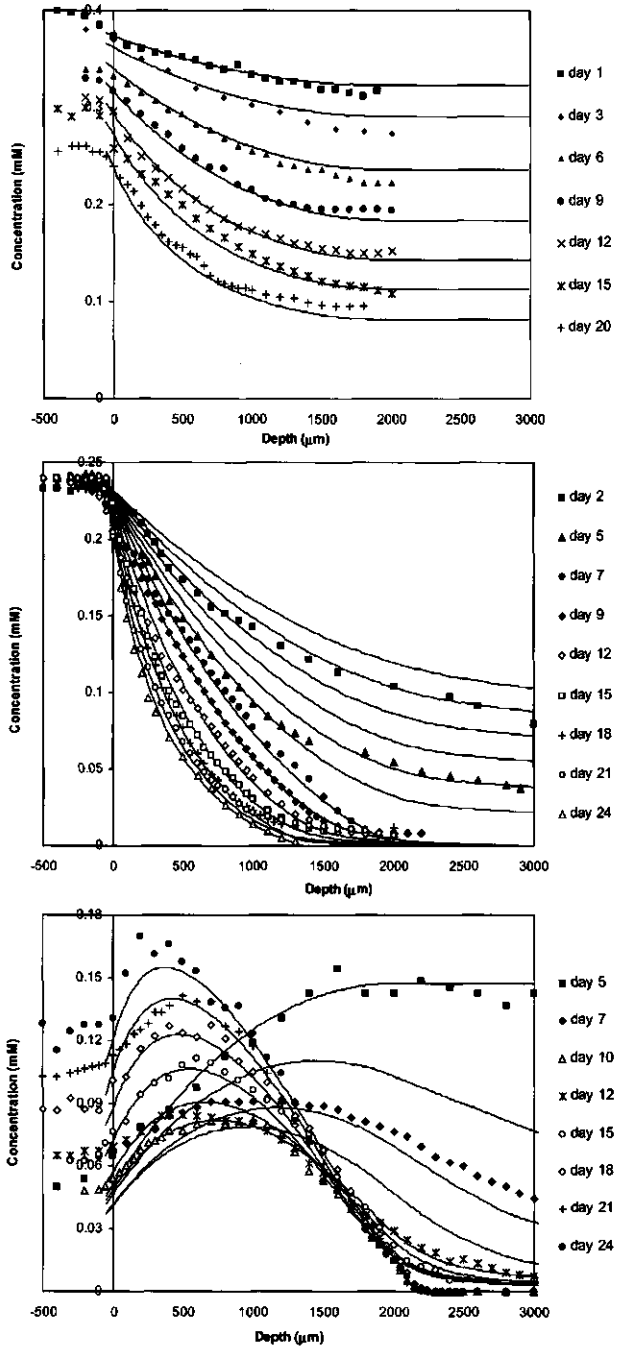


Figure 5 – Time-evolution of the predicted (lines) and measured (points) transient-state ammonium (A), oxygen (B) and nitrite (C) profiles across the double-layered gel slab under nitrifying and coupled nitrifying/denitrifying conditions.

Concomitantly, the ammonium bulk concentration declined with time whereas that of nitrite increased, reflecting the raise in the overall nitrifying activity. The shift from predominantly nitrifiying to simultaneously nitrifying and denitrifying conditions becomes evident namely from the change of shape of the nitrite profiles within the experiment time-span.

As a result of the profile development, the bulk of the nitrifying activity progressively concentrated to a narrower zone within the nitrifying layer and towards the end of the experiment nitrification took place basically within the first 700 to 900 μm from the slab surface.

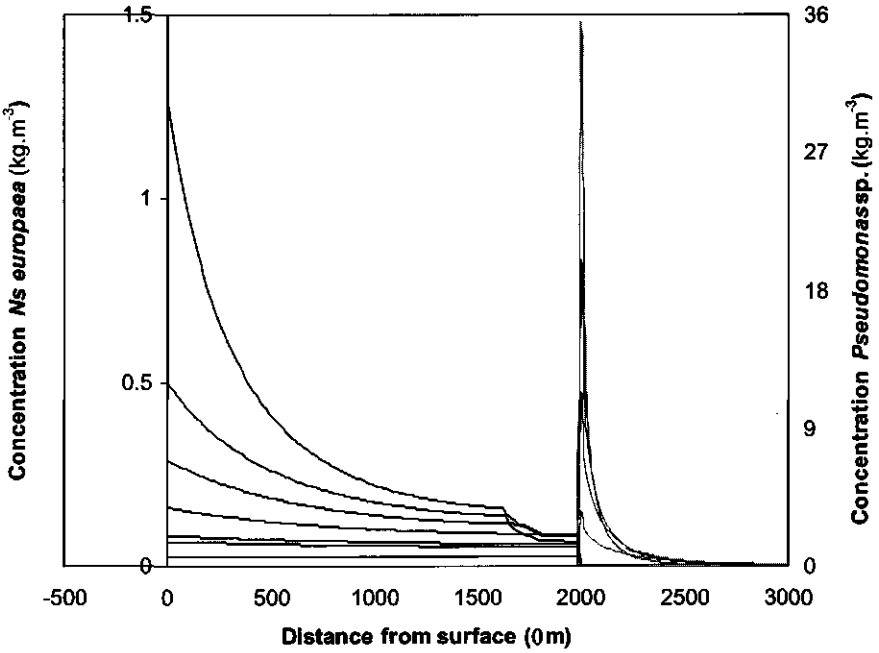


Figure 6 - Predicted biomass profiles across the gel slab as a function of time under coupled nitrification and denitrification conditions.

This connection to cell growth as well as its influence on these gradients is well understood by analyzing Figures 5 in close relation to Figure 6, which shows the predicted profiles for nitrifying (solid line) and denitrifying (dotted line) cells for the whole experiment. At the beginning, both nitrifying and denitrifying cells are homogeneously distributed throughout the outer and inner gels slab, respectively. As growth proceeds, however (and initially only for nitrifiers due to the absence of an

external C-source), the cells closer to the surface are the first to use the supplied substrates (whose availability is given by the Monod term introduced above). Since these substrates are only scarcely supplied (in particular ammonium) the nitrifying cells in the inner regions begin to suffer from substrate limitation and start to grow at lower rates. On the medium term, this results in the development of both substrate (Figure 5) and biomass (Figure 6) gradients across the outer slab. Owing to their easier access to the limiting substrates, the outer cells grow at higher rates and limit thereby even more the amount of substrate that is available for the inner cells. This in turn leads to further sharpening of these profiles so that at the long term nitrification would take place only in the outermost layers of the nitrifying slab. The development of such nitrifying profiles in gel matrices as a result of diffusion limitation has been thoroughly demonstrated earlier (Wijffels, *et al.*, 1991, Hunik *et al*, 1994). Hence, although these biomass profiles were not verified experimentally in this study, it is likely that they represent a reasonably realistic view of the actual biomass distribution as its is suggested by the good agreement between measured and predicted substrate and product profiles.

***Heterotrophic oxygen consumption***

As referred to above, in the experiment shown in Figure 4A there was a considerable discrepancy between the predicted (line) and measured (diamonds) oxygen profiles. Since the kinetics of oxygen consumption of this very same *Nitrosomonas europaea* strain under several nitrifying conditions has been studied into great detail previously (Hunik *et al.*, 1992, 1994), it was not likely that such deviation would result from an ill-defined stoichiometry. In fact, as it was suggested above, this was most probably caused by the heterotrophic activity of *Pseudomonas* in the denitrifying layer (Figure 5) at the expenses of internal carbon pools.

Under coupled nitrification and denitrification conditions (Figure 4B) there was no significant deviation between predicted and measured oxygen profiles because the model takes already into account the alternative use of oxygen or nitrite (depending on its availability) by *Pseudomonas* sp (a known facultative heterotroph) as electron acceptor in the presence of organic matter (Robertson *et al.*, 1990).

*Storage of organic carbon*

A remarkable observation was that coupled nitrification and denitrification seemed to take place for more than a week after stopping the supply of acetate. Figure 7shows a nitrite profile across the slab measured 6 days after having shifted from coupled nitrifying / denitrifying conditions in the reactor (measured in Figure 4B) to purely

nitrifying ones again. If only nitrification would have taken place then the shape of the profile should have been similar (possibly sharper) to that of Figure 4A. Yet, that was clearly not so and nitrite was being consumed in the inner part of the slab in a similar way as in Figure 4B. Similar profiles (though reflecting progressively less denitrification) were measured up to a total of ten days after the shift.

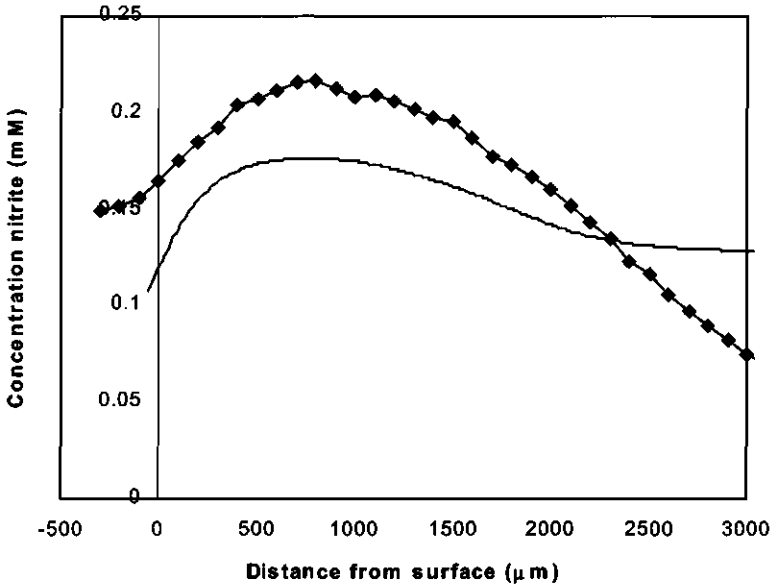


Figure 7 – Predicted (solid line) and measured (symbols) nitrite profile across the gel slab under purely nitrifying conditions measured 6 days after stopping the supply of acetate in the experiment whose results are shown in Figure 4.

Although it is theoretically possible that denitrifiers would have been active by using cell debris as a result of starvation, it is much more likely that they would have built up significant amounts of storage polymers (e.g. poly-β-hydroxyalkanoates (PHA)) during their growth under heterotrophic conditions. Indeed, such observations have been reported for many microorganisms (including several *Pseudomonas* strains) as a result of changing environmental conditions and following "feast-famine" regimes as a way of balancing the internal cell metabolism (Van Loosdrecht *et al.*, 1997, Wanner & Egli, 1990, Zevenhuisen *et al*, 1974, Van Aalst-Van Leeuwen *et al*, 1997, Doi, 1990).
According to Van Aalst-Van Leeuwen *et al* (1997), PHA's such as poly(β-hydroxybutyrate) (PHB) would be formed directly from the central intermediate (acetyl-CoA) of the cell metabolism of carbon substrates (such as acetate). The cell would therefore use PHB as energy and carbon source if the external substrate would be

exhausted. Clearly, this would explain both the "extra" oxygen uptake of Figure 4A and the long-lasting denitrification under autotrophy of Figure 7. The model, however, was clearly unable to handle such behavior and thorough modifications (possibly by building up a structured model instead) would have to be made to account for these effects.

*Cell decay*

To assess the survival capacity of *Pseudomonas* sp. under nutrient (acetate) depletion, a biomass decay experiment was done by measuring the number of viable cells along time in absence of an external carbon source. The outcome is shown in Figure 8.

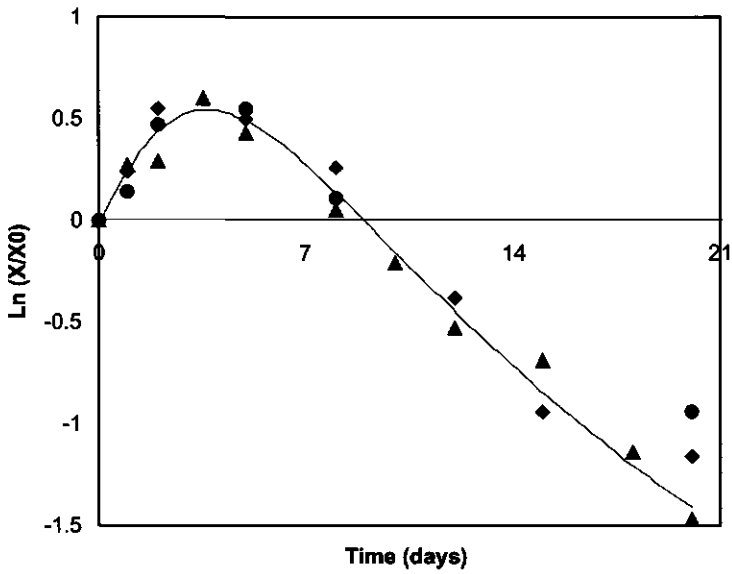


Figure 8 – Death (and growth) of *Pseudomonas* sp. in time in the absence of an external organic C-source. The solid line is a non-linear fit to the data to facilitate visual analysis.

Instead of decreasing, the cell number increased (at an initial rate of about $2.7 * 10^{-6}$ $s^{-1}$) within the first three to four days after start up. Thereafter, it declined at a more-or-less constant rate of $1.6 * 10^{-6}$ $s^{-1}$. These rates represent 9.7 % and 5.6%, respectively, of the maximum growth rate on acetate reported for this strain under aerobic conditions ($2.78 * 10^{-5}$ $s^{-1}$, Robertson *et al.*, 1990). As a comparison note that, according to the model of Beeftink *et al.* (1990) for cell growth at very low substrate concentrations (in which the decay rate of biomass (Kd) is determined by the product of the maintenance and the yield coefficient, see also Leenen *et al.*, 1997), we come up with a decay rate of $3.8 * 10^{-6}$ $s^{-1}$, which is 14% of $\mu_{max}$ and about three times higher than the measured decay of

1.6 * $10^{-6}$. These data support the above-mentioned hypothesis of PHA accumulation under intermittent substrate supply as suggested by Van Loosdrecht *et al.* (1997) and Van Aalst-Van Leeuwen *et al.* (1997). These findings are especially relevant with regard to the application of such systems to wastewater purification because the intervening cells seem thus able to face better the highly dynamic conditions characteristic of common treatment processes. This knowledge, together with that obtained by Leenen *et al.* (1997) on the effect of biomass decay on nitrification, will certainly enlarge our understanding on the dynamics of nitrogen removal by immobilized cells and will therefore allow us to design and optimize more effective and compact N-elimination processes.

## CONCLUSIONS AND PERSPECTIVES

In this study, we aimed at extending our knowledge and understanding of the mechanisms underlying nitrification, denitrification and coupled nitrification and denitrification by co-immobilized cells in a compact reactor system. The predictions yielded by a dynamic model earlier developed for describing integrated nitrification and denitrification by co-immobilized bacteria were compared to experimentally determined (by means of specific microelectrodes) substrate and product gradients across a double-layered biocatalyst under several operating conditions. Under all circumstances (i.e. denitrification only, nitrification only and coupled nitrification and denitrification), the predicted $O_2$, $NH_4^+$ and $NO_2^-$ microprofiles matched well the gradients measured experimentally. Also, the transitions from one set of conditions to the other were in all cases properly accounted for by the model, which reflects the appropriateness of this model to describe the dynamic behavior of the system. Such a mechanistic model is thus not only a valuable tool for the design and development of each of the N-removal processes outlined above and under given steady conditions but also for the more general case of strongly changing environments, which is the most frequent one in wastewater treatment practices.

To our knowledge [and as a follow-up to earlier work at our group on (de)nitrification by immobilized cells (Wijffels *et al*, 1991,1994, 1995, 1996, Hunik *et al.*, 1992, 1994, Leenen *et al*, 1997, Martins dos Santos *et al*, 1996a,b, *2001*a,b)], this is the first report on a detailed study of simultaneous nitrification and denitrification by co-immobilized bacteria that is supported by both a sound theoretical framework (dynamic mechanistic model) and an accurate experimental technique (set of specific microsensors) to describe these processes at biocatalyst level. We expect that the insight and understanding gained by this series of studies into the complex mechanisms underlying integrated nitrogen removal by (co-)immobilized microorganisms in its various aspects,

will provide a sound and thorough scientific basis for the design, control and scale up of effective and efficient treatment systems for the compact elimination of nitrogen from wastewaters. In this regard, and by making extensive use of this newly acquired knowledge, we have recently reported on the design and optimization of a compact, fully autotrophic system for nitrogen removal based on such immobilized-cell technologies (Martins dos Santos, *2001*a,b).

**ACKNOWLEDGEMENTS**

We thank Harald Ruissenaars and Herman Krab (Wagenigen University) for their help in preliminary experiments. We acknowledge the financial support given by the Junta Nacional de Investigação Científica e Tecnológica (JNICT), Portugal.

**NOTATION**

| | | |
|---|---|---|
| $D$ | Dilution rate of the reactor | [$s^{-1}$] |
| $i$ | Component | [-] |
| $m$ | Microorganism | [-] |
| $D_{gel}^{i}$ | Diffusion coefficient component i in the gel | [$m^2.s^{-1}$] |
| $D_{w}^{i}$ | Diffusion coefficient component i in water | [$m^2.s^{-1}$] |
| $\delta$ | Thickness of boundary layer | [μm] |
| $k_{l,g}^{i}$ | Gas-liquid mass-transfer coefficient component i | [$m.s^{-1}$] |
| $k_{l,s}^{i}$ | Liquid-solid mass-transfer coefficient component i | [$m.s^{-1}$] |
| $K_{i}^{m}$ | Monod constant of *m* for i | [$mol.m^{-3}$] |
| $M_{i}^{m}$ | Maintenance of *m* on i | [mol. $kg^{-1}$ $s^{-1}$] |
| $R$ | Radius to the centre of the bead | [m] |
| $R_{max}$ | Maximum radius of the gel bead | [m] |
| $R_s$ | Substrate consumption rate | [$mol.m^3.s^{-1}$] |
| $R_x$ | Growth rate of the bacteria | [$kg.m^{-3}$.s-1] |
| $R_{s}^{sus}$ | Substrate production rate by suspended bacteria | [$mol.m^{-3}.s^{-1}$] |
| $R_{x}^{sus}$ | Production rate of suspended biomass | [$kg.m^{-3}.s^{-1}$] |
| $S$ | Substrate concentration | [$mol.m^{-3}$] |
| $S'$ | Limiting substrate concentration | [$mol.m^{-3}$] |
| $S_{bulk}$ | Substrate concentration in the bulk phase | [$mol.m^{-3}$] |
| $X$ | Concentration of biomass | [$kg.m^{-3}$] |
| $X^{m}_{bulk}$ | Concentration of suspended biomass *(m)* | [$kg.m^{-3}$] |
| $X^{m}_{max}$ | Max. biomass conc. in the gel microorganism (m) | [kg.m-3] |
| $Y^{m}_{i}$ | Yield microorganism (m) on substrate (i) | [kg/mol i] |
| $\mu^{m,i}_{max}$ | Maximum growth rate of (m) on (i) | [$s^{-1}$] |

## REFERENCES

Amann R, Glockner FO, Neef A. 1997. Modern methods in subsurface microbiology: in situ identification of microorganisms with nucleic acid probes. FEMS Microbiol Rev 20: 191-200

Amann R, Kuhl M. (1998). In situ methods for assessment of microorganisms and their activities. Curr. Opin Microbiol 1 (3): 352-358

Axelsson, A., Westrin, B. and Loyd D. 1991. Application of the diffusion cell for the measurement of diffusion in cells. Chem. Eng. Sci. 46:913-915

Beefink H.H., van der Heijden R.T.J.M., Heijnen J.J. (1990). Maintenance requirements: energy supply from simultaneous respiration and substrate consumption. FEMS Microbiology Ecology vol.73:.203-210°

Beuling, E. E., Vand den Heuvel, J.C., Ottengraf, S.P.P. Determination of biofilm diffusion coefficients using microelectrodes. *In*: R.H. Wijffels, R. Buitelaar, H. Wessels, J. Tramper and C. Bucke (Eds) Immobilized Cells: Basics and Applications. Elsevier Science, Amsterdam, The Netherlands. pp. 31- 38

Bird RB, Stewart WE, Lightfoot EN. 1960. Transport phenomena. Wiley International Edition, New York, USA.

Bryers JD, Banks MK. Assessment of biofilm ecodynamics: In: Physiology of Immobilized cells. de Bont, J., Visser, J., Matiasson, M., Tramper, J. (Eds.). 1990. Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 49-61.

Christensen PB, Nielsen LP, NP Revsbech, Sorensen J. 1989. Microzonation of denitrification activity in stream sediments as studied with a combined oxygen and nitrous oxide microsensor. Appl. Environm.Microbiol. 55:1234-1241

Cronenberg CCH., Van den Heuvel JC. 1991. Determination of glucose diffusion coefficients in biofilms with mocroelectrodes. Biosens. Bioelectron., 6:255-262

Dalsgaard T, de Zwart J, Robertson LA, Kuenen JG, Revsbech N-P. 1995. Nitrification, denitrification and growth of artificial Thiosphaera pantotropha biofilms as measured with a combined microsensor for oxygen and nitrous oxide. FEMS Microbiol. Ecol. 17:137-148

Damgaard LR, Larsen LH, Revsbech NL. 1995. Microscale biosnesors for environmental monitoring. Trends in Anal. Chem. 14:300-303

de Beer D and J. C. van den Heuvel. 1988. Response of ammonium-selective microelectrodes based on the neutral carrier nonactin, Talanta, 35:728-730.

de Beer D, van den Heuvel JC, Ottengraaf SPP.1993. Microelectrode measurements of activity distribution in nitrifying bacterial agreggates. Appl. Environ. Microbiol. 59:573-579

de Beer D, Schramm A, Santegoeds CM, Khul M. 1997. A nitrite microsensor for profiling environmental biofilms. Appl. Environm. Microbiol. 63:973-977

de Gooijer CD, Wijffels RH, Tramper J. 1991. Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in carrageenan: part 1. Dynamic modeling. Biotechnol. Bioeng. 38:224-231.

Doi Y. 1990 Microbial polyestersVCH Publishers, New York, USA

Heijnen JJ, van Loosdrecht MCM, Mulder A., Tijhuis, L. 1992. Formation of biofilms in a biofilm air-lift suspension reactor. Wat. Sci. Technol.26:647-654

Hellendoorn L, Ottengraaf SP,.Pennings JAMM, van den Heuvel JC, Martins dos Santos, VAP, Wijffels RH. 1999. Kinetic Behaviour of and Performance of a Co-immobilized System of Amyloglucosidase and *Zymomonas mmobilis*. Biotechnol. Bioeng. 63:694-704

Hooijmans CM,Gerats SGM , van Niel EWJ, Robertson RA, Heijnen JJ, Luyben KchAM. 1990a. Determination of growth and coupled nitriifcation / dewnitrification by immobilized Thiophaera pantotropha using measurementss and modeling oxyegn profiles. Biotechnol. Bioeng. 36:931-939.

HooijmansCM,Gerats SGM Potter JJM, Luyben KchAM. 1990b. Experimental determination of mass transfer boundary layer around a spherical biocatalyst particle. Biochem. Eng. Journal 44:B41

Hunik J.H. Tramper J., Meijer H.J.G. (1992). Kinetics of *Nitrosomonas europaea* at extreme substrate, product and salt concentrations. Appl. Microb. and Biotechnol. 37:802-807.

Hunik J.H., Bos C.G., Van Den Hoogen P., De Gooijer C.D., Tramper J. (1994). Co-immobilized *Nitrosomonas euroapaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in k-carrageenan gel beads. Biotechnol. Bioeng.

Hulst AC, Hens HJH, Buitelaar RM, Tramper J. 1989. Determination of the effective diffusion coefficient of oxygen in gel materials in relation ot concentration. Biotechnol Techniques 3:199-204

Jensen K, Revsbech NP, Nielsen LP. 1993. Microscale distribution of nitrification activity in sediment determined with a shielded microsensor for nitrate. Appl. Environm. Microbiol. 59:3287-3296

Jensen K, Sloth NP, Risgaard-Petersen, Rysgaard S, Revsbech NP. 1994. Estimation of nitrification and denitrification from microprofiles of oxygen and nitrate in model sediment systems. Appl. Environm. Microbiol. 60:2094-2100

Kurosawa H, Tanaka H. Advances in immobilized cell culture: development of a co-immobilized mixed culture system of aerobic and anaerobic micro-organisms. Process Biochemistry Int. 1990; 25: 189-196

Lee N, Nielsen PH, Andreasen KH, Juretschko S, Niesen JL, Schleifer K-H, Wangner M. 1999. Combination of fluorescent in situ hybridization and microautoradiography: a new tool for structure- function analysis in microbial ecology. Appl. Environ. Microbiol. 65:1289-1297

Leenen EJTM, Boogert AA, van Lammeren AAM, Tramper J, Wijffels RH. 1997. Dynamics of artificially immobilized *Nitrosomonas europaea:*effect of biomass decay. Biotechnol. Bioneg. 55:630-641

Lens P., de Beer D, Cronenberg C, Ottengraf S, Verstaete W. 1995. The use of microsensors to determine population distributions in UASB aggregates. Wat Sci. Technol. 31(1):273-280

Lide, D.R. (Ed.). 1992. Handbook of Chemistry and Physics. 73rd edition. CRC Press, Boca Raton, U.S.A.

Lorenzen J, Larsen LH, Kjaer T, Revsbech N-P. (1998). Biosensor for the determination of the microscale distribution of nitrate, nitrate assimilation, nitrifcation and denitrification in a diatom-inhabiuted freshwater sediment. Appl. Environm. Microbiol. 64:3264-3269

Lide, D.R. (Ed.). 1992. Handbook of Chemistry and Physics. 73rd edition. CRC Press, Boca Raton, U.S.A.

Mankad T, Nauman EB. (1992). Modeling of microbial growth under dual limitations. Chem. Eng. J. 48:B9-B11

Mackie, J.S, Meares, P. 1955. The diffusion of electrolytes in a cation-exchange resin membrane. Theoretical. Proc. Roy. Soc. London. A232, 498-509

Martins dos Santos V.A., Tramper J., Wijffels R.H. (1993). Simultaneous nitrification and denitrification using immobilized micro-organisms. Biomat., Art. Cells & Immob. Biotech., 21(3),317-322.

Martins dos Santos, V.A.P., Tramper, J., R.H. Wijffels. 1996a. The Magic Bead Concept: An Integrated Approach for Nitrogen Removal with Co-immobilized Microorganisms. Appl. Microbiol. Biotechnol. 45:447-453.

Martins dos Santos, V.A.P., L. Marchal, Tramper, J., R.H. Wijffels. 1996b. Modelling and Evaluation of an Integrated Nitrogen Removal System with Co-immobilized Microorganisms. Biotechnol. Prog. 12:163-171

Martins dos Santos, V.A.P., Jacobs M, Tramper, J., M. Jetten, G. Kuenen R.H. Wijffels. 2001a. Simultaneous Autotrophic Nitrification and Anaerobic Ammonium Oxidation with Co-immobilized Micro-organisms. Appl. Environm. Microbiol., submitted

Martins dos Santos, V.A.P., Verschuren, P., van den Heuvel, H., Tramper, J., Wijffels, R. *2001b.* pH effects on coupled nitrification and denitrification measured by specific microelectrodes. Biotechnol. Bioeng., submittted

Okabe S, Hiratia K, Ozawa Y, Watanabe Y. 1996. Spatial microbial distribution of nitrifiers and heterotrophs in mixed-populations biofilms. Biotechnol. Bioeng. 50:24-35.

Okabe S, Satoh H, Watanabe Y. 1999a. In-situ analyis of nitrifying biofilms as determined by in-situhybridization and the use of microelectrodes. Appl. Environm. Microbiol. 65:3182-3191.

Okabe S, Itoh T, Satoh H, Watanabe Y. 1999b. Analysis of spatial distributions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. Appl. Environm. Microbiol. 65:5107-5116

Ramsing NB, Khul M, Jorgensen BB. 1993 Distribution of sulfate-reducing bacteria, O2, and H2S in photosysnthetic biofilms determined by oligonucletide probes and microelctrodes. Appl. Environm. Microbiol. 59:3840-3849.

Revsbech N.P., WardD.M. 1980. Oxygen microelectrode that is insensitive to medium chemical composition: use in an acid microbial mat dominated by *Cyanidium caldarium.* Appl. Environm. Microbiol. 45:755-759

Revsbech NP, Christensen PB, LP Nielsen, Sorensen J. 1989. Denitriifcation ina trickling filter biofilm studied by a microsensor for oxygen and nitrous oxide. Wat. Res. 23:867-871

Revsbech NP, Christensen PB, LP Nielsen, Sorensen J. 1988. Combined oxygen and nitrous oxide microsnesor for denitrification studies. Appl. Environm. Microbiol. 54:2245-2249

Robertson L.A. & Kuenen J.G. (1990). Combined heterotrophic nitrification and aerobic denitrification in *Thiosphera pantotropha* and other bacteria. Antonie van Leeuwenhoek 57:139-152

Santegoeds CM, Schramm A, de Beer D. 1998. Microsensors as a tool to determinecchemical microgradients and bacterial activity in wastewater biofilms and flocs. Biodegardation 9:159-167

Santegoeds CM, Damgaard LR, Hesselink G, Zopel HJ, Lens P, Muyzer G, de Beer D. 1999. Distribution of sulfate-reducing and methanogenic bacteria ina anaerobic aggregates determined by microsensor and molecular analyses. Appl. Environm. Microbiol. 65:4618-4629

Schaller U, Bakker E, Spichiger UE, Pretsch E. 1994. Nitrite-selective microelectrodes, Talanta, 41:1001-1005.

Schramm A, Larsen LH, Revsbech NP., Ramsing NB, Amann R, Schleifer K-H. 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl. Environm. Microbiol. 62:4641-4647

Schramm A, de Beer D, Van den Heuvel JC, Ottengraf S, Amann R. 1999a. Microscale distribution of populations and activities of Nitrosospira and Nitrospira along a macroscale gradient in a nitrifying bioreactor: quantification by insitu hybridization and the use of microsensors. Appl. Environm. Microbiol. 65:3690-3696

Schramm A, Santegoeds CM, Nielsen HK, Wagner M, Pribyl M, Wanner J, Amann R, de Beer D. 1999b. On the occurrence of anoxic microniches, denitrification and sulfate reduction in aerated activated sludge. Appl. Environm. Microbiol. 65:4189-4196

Sherwood, T.K.A., Pigford, R.L., Wilke, C.R. 1975. Mass transfer. McGraw-Hill Book Company, London, UK

Sweerts JPRA, de Beer D. 1989. Microelectrode measurements of nitrate gradients in the littoral and profundal sediments of a meso-eutrophic lake (Lake Vechten, the Netherlands). Appl. Environm. Microbiol. 55:754-757

Sweerts JPRA, de Beer D, Nielsen LP, Verdow H, JC van den Heuvel, Cohen Y, Cappenberg TE. 1990. Denitrification by sulphur oxidizing Beggiatoa spp. mats on freshwater sediments. Nature 344:762-763

Sherwood, T.K.A., Pigford, R.L., Wilke, C.R. 1975. Mass transfer. McGraw-Hill Book Company, London, UK

Sweerts JPRA, de Beer D. 1989. Microelectrode measurements of nitrate gradients in the littoral and profundal sediments of a meso-eutrophic lake (Lake Vechten, the Netherlands). Appl. Environm. Microbiol. 55:754-757

Sweerts JPRA, de Beer D, Nielsen LP, Verdow H, JC van den Heuvel, Cohen Y, Cappenberg TE. 1990. Denitrification by sulphur oxidizing Beggiatoa spp. mats on freshwater sediments. Nature 344:762-763

Tijhuis L, van Loosdrecht MCM, Heijnen JJ. 1994. Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. Biotechnol. Bioeng. 44:595-608

Tramper, J. (1984) Nitrification and denitrification by immobilized bacteria, Proc. 3rd European Congress on Biotechnology, VCH, Vol. IV :363-368.

Tsien RY, Rink TJ. 1980. Biochim. Biophys Acta 599:623-628. Neutral carrier ion-selective microelectrodes for the measurement of intacellular free calcium.

van Benthum WAJ, van Loosdrecht MCM, Heijnen JJ.1997. Control of heterotrophic layer formation on nitrifying biofilms in a biofilm airlift suspension reactor. Biotechnol. Bioeng. 53:397-405.

van Aals-van Leeuwen, MA, Pot MA, Van Loosdrecht MCM, Heijnen JJ. (1997) Kinetic modelling of poly(β-hydroxybutyrate) production and consumption by Paracoccus pantotrophus under dynamic substrate supply. Biotechnol. Bioneng. 55:773782

van Loosdrecht MCM, Pot MA, Heijnen JJ. (1997). Importance of bacterial storage polymers in bioprocesses. Wat. Sci. Technol. 35(1):41-47

Wanner U, Egli T. 1990. Dyanamics of microbial growth and cell composition in batch culture. FEMS Microbial rev. 75:19-44

Westrin BA. Diffusion measurements in gels: a methodological study. PhD dissertation Lund University, Lund, Sweden.

Wijffels RH and Tramper J. Nitrification by immobilized cells. 1995. Enz. Microb. Technol., 17:482-492

Wijffels RH, Eekhof MR, Van den Heuvel, JC, Tramper J. 1995. Pseudo-steady-state oxygen concentration profiles in an agar slab containing growing *Nitrobacter agilis*. J. Ferment. Bioeng. 2:167-170

Wijffels R.H., De Gooijer C.D., Kortekaas S., Tramper J. Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in k-carrageenan. Part 2: Model evaluation. 1991. Biotechnol. Bioeng. 38: 232-240

Wijffels R.H., Schepers A.W., Smit M., de Gooijer C.D., Tramper J. 1994. Effect of initial biomass concentration on the growth of immobilized *Nitrosomonas Europaea*. Appl. Microb. Biotech. 42:153-157

Wijffels R.H., de Gooijer C.D., Schepers A.W., Beuling E.E., Mallée L.F., Tramper J. 1996. Dynamic Modeling of immobilized Nitrosomonas Europaea: implementation of diffusion limitation over expanding micro-colonies. Enz. & MIcrob Tech. 17:462-471

Zevenhuisen LPTM, Ebbink AG. 1974. Interrelations between glyvcogen, poly-β-hydroxybutyric acid and lipids during accumulation and subsequent utiliation in a *Pseudomonas*. Ant. Van Leeuwen. 40:103-120.

Zhang TC, Bishop P. 1996. Evaluation of substrate and pH effects in a nitrifying biofilm. Wat. Environm. Res.68:1107-1115.

Zhang TC, Y-C Fu, Bishop P. 1995. Competition for substrate and space in biofilms. Wat. Environm. Res. 67:992-1003

**APPENDIX** To determine the "most-limiting" substrate the Monod terms of the substrates involved are calculated. According to the non-interactive model (Mankad & Nauman, 1992) the specific growth rate of a micro-organism is limited by a single substrate at the time. The limiting substrate is that for which the Monod term is the smallest. The specific growth rate is therefore given by:

$$\mu = \mu_{max} min\left( \frac{S^1}{S^1 + K_{S^1}}, \frac{S^2}{S^2 + K_{S^2}} \right) \tag{1}$$

When the "most" limiting substrate ($S^i$) for a microorganism (m) has been determined, the rate ($r_S$) of substrate utilization is given by:

$$-r_s = \frac{dS}{dt} = \left( \frac{\mu^m{}_{max}}{Y_{m,s}} + m^m{}_s \right) \left( \frac{S^i}{S^i + K_{S^i}} \right) X^m \tag{2}$$

The biomass production rate for microorganism (m) and limiting substrate ($S^i$) is therefore defined as:

$$r_X{}^m = \frac{dX^m}{dt} = \mu^m{}_{max} \left( \frac{S^i}{S^i + K_{S^i}} \right) X^m - m_s{}^m X^m Y_s{}^m \left( 1 - \frac{S^i}{S^i + K_{S^i}} \right) \tag{3}$$

Applying a differential mass balance over the biocatalyst slab for simultaneous diffusion and consumption of substrate we come at:

$$\frac{\partial S^i}{\partial t} = D^i{}_{gel} \left( \frac{\partial^2 S^i}{\partial x^2} \right) - r_s \tag{4}$$

with boundary conditions:

$$\text{for x = 0} \qquad D^i_{gel}\frac{\partial S^i}{\partial x} = 0$$

$$\text{for x = L} \qquad D^i_{gel}\frac{\partial S^i}{\partial x} = k^i_{l,s}\left(S^i{}_{bulk} - S^i{}_{surf}\right)$$

The model is based on the assumption of an ideally mixed reactor thus $S_{out}$ is equal to $S_{bulk}$. The corresponding mass balances over the reactor are thus given by:

$$\frac{dX^m{}_{bulk}}{dt} = D\left(X^i_{in_{in}} - X^i{}_{bulk}\right) + r^{susp}_{X^m} \tag{5}$$

$$\frac{dS^i_{bulk}}{dt} = D(S^i_{in} - S^i_{bulk}) - r^{susp}_s - k^i_{l,s}A\left(S^i_{bulk} - S^i_{surf}\right) \tag{6}$$

Eqs. 5 and 6 include terms corresponding to substrate consumption by suspended biomass ($r_S{}^{susp}$) and bacterial growth in the bulk ($r_X{}^{susp}$) as a result of released cells.

This chapter has been submitted for publication by Martins dos Santos VAP, Verschuren PG, Van den Heuvel JC, Tramper J, Wijffels RH.

# CHAPTER 9

## pH Effects on Coupled Nitrification and Denitrification Predicted by a Dynamic Model and Measured by Specific Microelectrodes


### ABSTRACT

In this work, we describe both a conceptual framework and a detailed experimental analysis of the pH effects on coupled nitrification and denitrification. A previously validated dynamic model describing coupled nitrification and denitrification within immobilization matrices was extended to account for pH effects on microbial activity. The model was verified by measuring profiles of oxygen, ammonium, nitrite and pH across the support and along time under a wide set of conditions for nitrification, denitrification and coupled nitrification and denitrification. The influence of the bulk pH, buffer capacity and buffering effect of the initial substrate was studied as well. The results obtained and the data generated have clearly shown that coupling nitrification and denitrification within a single catalytic particle is an effective means of minimizing inhibition of microbial activity by pH. Due to the partial compensation of acidification resulting from nitrifying activity by alkalization) due to denitrifiers, a much more robust multistep process is attained. This has great significance for the practice of waste and industrial water treatment and greatly facilitates pH control, which requires much less buffer capacity than for conventional systems. Hence, information on the specific sensitivity of nitrifiers or denitrifiers to pH, together with the knowledge of the influence of different factors on pH development (buffer capacity, bulk pH and so forth) as well as the time scale for pH development yield, as a whole, a broad framework for predicting and handling pH effects on the performance of nitrogen removal systems. This will ultimately provide tools for more effective design and control of treatment processes of waste and industrial waters.

## INTRODUCTION

The steadily tightening of the limit-values for total-nitrogen in wastestreams imposed by many regulatory institutions has been stimulating a strong demand for new approaches on nitrogen removal technology. Such demands aim at developing effective processes that minimize both space and energy requirements while meeting the strict environmental regulations (see Martins dos Santos *et al.*, 1998a for a review). The trends set forth during the last decade show that most of the newly developed technologies rely on immobilized-cell processes because the nitrogen removal rates attained can be very high in rather compact reactors. These processes, either based on artificially immobilized cells (Wijffels *et al.*, 1995, Martins dos Santos, 1992, 1996, Tanaka *et al.*, 1996) or naturally attached biofilms (Tijhuis 1994, Van Benthum *et al.* 1995, Heijnen *et al.*, 1991), have in common that the intervening microorganisms experience important restrictions on substrate and product transport over the liquid-phase interface and throughout their immobilization matrix. These restrictions lead to the development of steep gradients of both solute and biomass, which will ultimately determine the overall performance of such process (see also Willaert *et al.* (1996)).

To gain insight into to the mechanisms underlying these processes, substrate, product and biomass gradients have been modeled for some of these systems (De Gooijer *et al.*, 1991, Tijhuis 1994, Picioreanu, 1998a,b, Hunik *et al.*, 1994), whereas for others the actual profiles were measured (Hunik *et al.*, 1993, 1994, Wijffels *et al.*, 1991, De Beer *et al.*, 1990, 1998, Martins dos Santos *et al.*, 2001). However, in all these reports, the possible effect of the local pH on growth and reaction rate within a nitrogen removal support has been strongly underplayed. Indeed, these studies have been done at lab scale, under carefully controlled conditions and with abundant buffering, which certainly minimizes possible pH effects. Yet, real-scale treatment processes will tend to rely on both the natural buffering capacity of the wastestreams and on the use of less chemicals possible for pH control. Hence, the optimal design and scale-up of compact nitrogen removal systems based on immobilized-cell technology demands a thorough knowledge of the processes involved in the onset of pH gradients throughout biocatalytic supports and, conversely, of the effects of these pH gradients on the local degrading activity and growth of the intervening microorganisms. This knowledge, however, is still limited because of the complex interdependencies resulting from the transport of all the intervening solutes, the conversion kinetics of each of these solutes within the support and the growth of the microorganisms involved. The first attempt to tackle these issues was done by Riemer and Harremöes (1978) by describing, theoretically, multicomponent diffusion in denitrifying biofilms using conventional

biofilm kinetics. Later, Arvin and Kristensen (1982) confirmed indirectly these predictions by showing that the pH of a denitrifying biofilm was considerably higher in the rear of the biofilm than in the bulk liquid. Similar approaches were followed later for nitrifying biofilms (Szwerinski *et al.*, 1986, Siegrist and Gujer, 1987). More recently, De Beer *et al.* (1993) were able to actually measure pH profiles throughout nitrifying aggregates at low buffering capacity using a pH microelectrode. However, although the pH profiles clearly reflected a strong nitrifying activity, they did not present a mechanistic model that predicted the development of such profiles.

Hence, the work presented in this paper aims at providing more insight into these issues for a nitrifying, denitrifying and simultaneously nitrifying-denitrifying system with autotrophic nitrifiers and heterotrophic denitrifiers co-immobilized in a double-layered gel support. A previously developed dynamic model incorporating simultaneous mass transfer, substrate consumption and growth (Martins dos Santos *et al.*, 1996b, 2001) is extended to include the development of pH profiles across the biocatalyst support. The buffering effects of the medium components as well as those resulting from conversion products are included. Model predictions were compared with the experimental profiles measured with $NH_4^+$, $NO_2^-$, pH and $O_2$ microelectrodes for nitrifying, denitrifying and nitrifying-plus-denitrifying conditions.

## MATERIALS AND METHODS

### Strains and immobilization procedure

*Nitrosomonas europaea* ATCC 19718 and *Pseudomonas* spp., strain LMD 84.60 (formerly *Pseudomonas denitrificans*) were cultivated, harvested, concentrated and immobilized in a double-layered agar slab as described before (Martins dos Santos *et al.*, 1996b, 2001). The slab had a diameter of 11.5 cm, the nitrifying layer (outer) was 2 mm thick and the denitrifying one (inner) was 3 mm.

### Immobilized-cell cultivation

The four-baffled vessel containing the double-layered agar slab was placed in an Faraday cage and was used as a continuous stirred submerged reactor as described previously (Martins dos Santos, 2001, see also Figure 1). The reactor medium (Martins dos Santos *et al.*, 1996b), supplied at a dilution rate of $5.56 * 10^{-4}$ $s^{-1}$ and having a pH of 7.3, contained 0.5 mM $NH_4^+$ under nitrifying conditions alone and, whenever convenient, was supplemented with 0.5 mM acetate to provide denitrifying conditions. For denitrification alone, ammonium was suppressed. Oxygen, ammonium and nitrite profiles across both layers of the plate were measured at different time steps both for

nitrifying and denitrifying conditions. For measurements under unbuffered conditions, a medium without phosphate salts was prepared. Oxygen was supplied to the reactor through a small air sparger at a rate of about 10 $ml.s^{-1}$. The reactor was maintained at 30°C and was stirred by an air-driven six-blade stirrer placed at 2 cm above the gel surface.

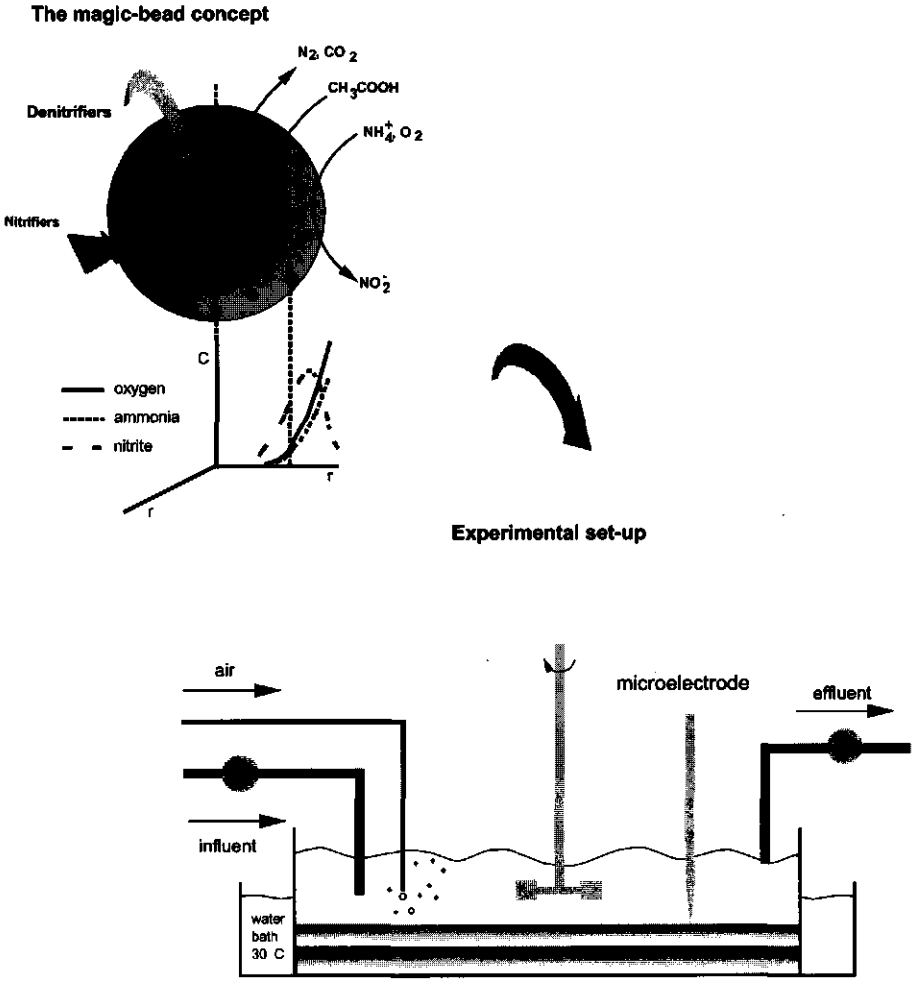


Figure 1 - Schematic representation of the Magic-bead concept and the experimental set up.

**Ion-selective and oxygen microelectrodes**

The nitrite, ammonium and oxygen electrodes were prepared and calibrated as described in Martins dos Santos *et al.*, (2001) on the basis of earlier work of De Beer (1988, 1992, 1993). The pH microelectrode (tip diameter 1μm) was prepared as described by de Beer *et al.* (1988).

**Other analytical methods**

Ammonia, nitrite and nitrate (as a control) concentrations in the influent and effluent of the continuous reactors were measured using a continuous-flow analysis system (Autoanalyser SB40, Skalar Methods) as earlier described (Hunik, 1994). Influent and effluent acetate concentrations were measured by gas chromatography as described before (Martins dos Santos *et al.*, 1996).

**MODELING**

**Base model**

The base model was reported previously for a simultaneously nitrifying-denitrifying system with autotrophic nitrifiers and heterotrophic denitrifiers co-immobilized in double-layered gel beads (Martins dos Santos *et al.*, 1996a). A mass balance for all components over the reactor was coupled to a biocatalyst model that describes diffusion of components, substrate utilization and growth, all occurring simultaneously. The overall energy/synthesis equations were (Martins dos Santos *et al.*, 1996):

$$NH_4^+ + 1.379\,O_2 + 1.986\,HCO_3^- \Rightarrow$$
$$0.983\,NO_2^- + 0.0191\,C_5H_7NO_2 + 1.041\,H_2O + 1.890\,H_2CO_3 \qquad (1)$$

for nitrification (carried out by *Nitrosomonas europaea)*, and

$$NO_2^- + 0.0654\,NH_4^+ + 0.537\,CH_3COOH + 0.184\,H_2CO_3 \Rightarrow$$
$$0.5\,N_2 + 0.0654\,C_5H_7NO_2 + 0.934\,HCO_3^- + 0.696\,H_2O \qquad (2)$$

for denitrification (acomplished by *Pseudomonas* sp). These equation account already for the changes in the carbonic-acid system. Mass transfer from the bulk to the cell support (and backwards) was calculated by the film theory. Macroscopic consumption and production rates were calculated according to Monod kinetics in which maintenance

was accounted for. The biocatalyst support was divided in 244 equally-spaced shells (as an analogy with a peeled onion) in which, at each time step, uniform conditions are assumed. For each shell, at each time step, "the most limiting substrate" of the several possible for each microorganism is chosen (for more details see Martins dos Santos *et al.*, 1996b and Hunik *et al.*, 1994). After doing this, the local substrate consumption and product formation rates are calculated and from these, the corresponding profiles across the whole support. These values are subsequently used, at each time step, to calculate local growth rates which will be in turn used for calculation of biomass profiles throughout the matrix. The cycle is repeated for every time step (either 20 or 50 s) until the simulated time is reached. The equations for growth of biomass are solved by a second order Runge-Kutta algorithm whereas those for the bulk concentrations of biomass and solute were solved by a procedure based on the trapezoidal rule. The solute concentration profiles across the matrix were solved through a backward-in-time and centered-in-space finite-differences algorithm for partial differential equations. The model thus, predicts biomass and solute bulk concentrations, substrate consumption rates, product formation rates, and biomass and solute concentration profiles within the beads as a function of time. Fluctuations in the substrate load, dilution rates or mass transfer parameters can be accommodated as well. Intrinsic kinetic parameters of the micro-organisms, internal and external mass transfer coefficients, initial conditions, bead concentration and particle diameters are the input parameters. This model was experimentally evaluated at both reactor (Martins dos Santos *et al.*, 1996a) and biocatalyst level (Martins dos Santos *et al.*, 1996b). Validation at biocatalyst level was done by comparing predicted substrate and product profiles across the cell supports under different operating conditions with those measured in beads and gel slabs with $NH_4^+$, $NO_2^-$, and $O_2$ microelectrodes (Martins dos Santos *et al.*, 2001).

**Extension to pH calculations**

The above-described model was extended to include the development of pH profiles and their effects on the substrate, product and biomass profiles across the biocatalyst. Hereto, mass balances for the species involved in the pH changes were added to the balances already made for the other products and substrates (Appendix I, see also Martins dos Santos *et al.*, 1996b). The final model would thus aim at calculating profiles for $NH_4^+$, $NO_2^-$, $CHCOO^-$, $H^+$, $HPO_4^{2-}$, $H_2PO_4^-$, $PO_4^{3-}$, $HCO_3^-$, $CO_3^{2-}$, $CO_2$ and $N_2$. However, the simple expansion of the calculation matrix for the solute concentration would lead to a substantial increase in computation time and a decrease in the maximum number of shells allowed (less resolution). To circumvent this difficulty the

concentration of each ionic species was expressed as a fraction of the total concentration of the species. Hence, for instance $H_2CO_3$, $HCO_3^-$ and $CO_3^{2-}$ were expressed as a fraction $\alpha_0$, $\alpha_1$, and $\alpha_2$ of the total inorganic carbon, $C_{Ti}$ (Flora *et al.*, 1993, Snoeyink and Jenkins, 1980). These fractions $\alpha_i$ are function of the pH and the dissociation constants of the carbonate and carbonic acid (see Notation and Appendix). By doing this for the other electrolytes, only one variable per electrolyte species was introduced directly in the matrix, reducing thereby substantially the load on computation.

Basically, the main differences of this model with respect to the base one described above is that i) the equilibrium constants for the weak acids and bases were taken into account and ii) a local alkalinity balance was made within each shell to guarantee electroneutrality. For the calculation procedure this meant basically that i) the calculation matrix for the solute concentration was slightly expanded and ii) an extra routine was introduced in which the local pH is calculated through a simple Runge-Kutta procedure as described by Lens *et al.*, 1993 and Cronenberg (1994).

In rigorous terms, the mass balances for the ionic species should include the electrostatic potential gradients that result from the charge interactions between ions (Flora *et al.*, 1993, 1995). However, the inclusion of such balances into the model would increase substantially its complexity and computation time. Since we operate at relatively low concentrations for all ions (< 10-20 mM) and low ionic strength we expect the magnitude of these electrostatic gradients to be negligible with respect to the pure chemical gradients. For that reason, we described the behavior of the electrolytes according to Fickian diffusion only and coupled this analysis to local electroneutrality balances.

**Dependence of the kinetic parameters on pH**

Tan and co-workers (1998) have developed a generalized statistical thermodynamic approach for modeling pH effects that contains in itself the existing models for pH inhibition (see Bailey & Ollis, 1986). The model assumes that growth is determined by the rate-limiting step of a given sequence of enzymatic reactions and therefore it explains pH effects in terms of ionization of the active components of microbial cells. We applied this technique here to assess the pH dependence of reaction rate using literature data for both *Nitrosomonas europaea* and *Pseudomonas* spp.

*Nitrosomonas europaea*

Hunik *et al.* (1992) have measured experimentally the effect of pH on both the maximum volumetric conversion rate and the affinity constant for ammoni(a)um of

*Nitrosomonas europaea*. On the basis of their data and of steady-state analysis of the hypothesized reaction mechanism for ammonium oxidation (Quinlan, 1984, Antoniou *et al.*, 1990), we found the following relation for the dependence of the maximum volumetric conversion rate ($r_s$, $mol.m^{-3}.s^{-1}$) on the pH:

$$\frac{r_s}{r_s^0} = \left[ \frac{1.08}{1 + 10^{6.51-pH} + 10^{pH-9.79}} \right] \tag{3}$$

$r_s^0$ is the rate at standard conditions ($NH_4^+$ concentration of 25 mM, pH 7.5 with 50 mM Tris/HCl and 50 mM phosphate buffer). The value 1.08 is slightly higher than one because the calculated optimal pH is 7.96 instead of 7.5 of the standard conditions. This relation is similar to that used by Hunik *et al.* (1992) for these same data and to those found by Quinlan (1984), Laudelout *et al.* (1976) and Suzuki *et al.* (1974) for ammonia oxidizers. For the affinity constant for ammonia at a given pH we found:

$$K_s = 8.03 * 10^{-2} \bullet \left[ \frac{1}{1 + 10^{6.91-pH} + 10^{(pH-9.19)}} \right] \bullet \left[ 1 + 10^{8.78-pH} \right] \tag{4}$$

Note that although the true substrate for *Nitrosomonas* is $NH_3$ (Suzuki *et al.*, 1974), the affinity constant is often expressed as total ammonia (i.e. $NH_4^+ + NH_3$). We did the same here and hence the last term of Eq. [2] represents the correction for total ammonia through the equilibrium constant. A relevant feature of this equation is that it shows that the affinity for ammonia actually increases for higher pH.

Based on experimental data and using an analogy between enzyme kinetics and kinetics of microbial growth, Antoniou *et al.* (1990) proposed the following expression (at 303K) for the dependence of the (net) specific growth of *Nitrosomonas* spp on pH:

$$\frac{\mu_{net}}{\mu_{net}^{\max}} = \frac{1.12}{1 + 10^{6.78-pH} + 10^{pH-8.69}} \tag{5}$$

The maximum net specific growth rate ($\mu_{net}$, here expressed in $d^{-1}$) is the difference between true growth and decay. For a pH of 7.7 (about their optimal pH) this expression yields a growth rate of 1.89 $d^{-1}$, which is slightly higher than that used here of 1.38 $d^{-1}$ but it is within those reported in literature, which vary widely (see also Hunik *et al.*, 1994). Note that this expression is very similar to that in Eq. (4). This is not surprising because growth rate and substrate consumption are related through yield and

maintenance (see Appendix B). Hence, this supports as well that the assumption that growth and its dependency of a rate-limiting enzymatic step is correct.

*Pseudomonas* spp (formerly *Pseudomonas denitrificans*).
Similarly, using the experimental values of Wang *et al.*, (1995) for *Pseudomonas denitrificans*, of Almeida *et al.* (1995) for *Pseudomonas fluorescens* and those of Thomsen *et al.* (1993) for *Paracoccus denitrificans*, similar expressions were obtained for the pH-dependency of $r_s$ and $K_s$ for nitrite:

$$\frac{r_s}{r_s^0} = \frac{1.09}{1+10^{6.9-pH}+10^{pH-7.2}} \tag{6}$$

and

$$K_s = 2.97 * 10^{-2} \bullet \left[\frac{1}{1+10^{7.32-pH}}\right] \bullet (1+10^{(3.32-pH)}) \tag{7}$$

respectively. Note that this relation for the affinity constant shows that affinity increases for decreasing pH. These equations (coupled with experimental yield and maintenance) resulted in a maximal net growth rate at pH 7.5 (2.88 $d^{-1}$), which agrees with that experimentally determined by Robertson *et al.* (1989) for the *Pseudomonas* spp strain (formerly *Pseudomonas denitrificans*) used in this study (kindly given by Dr. L. Robertson, Technical University Delft).

***Inhibition***

*Nitrosomonas europaea:* Several authors have shown that ammonium itself does not inhibit its own oxidation (Hunik *et al.*, 1992, Wiesmann, 1994, Sheintuch *et al.* 1995). Ammonia, however, inhibits microbial activity if present at high concentrations, as shown by Wiesman (1994) and Dombrowski (1991). They found that the oxidation activity as a function of the ammonia concentration followed a Haldane-Andrews kinetics and calculated an inhibition constant for $NH_3$ of 38.6 mM. Though this constant did not depend on the pH, ammonia concentration in the medium varies as a function of the pH (pKa=9.09 at 30°) and it is in equilibrium with ammonium. As in poorly buffered medium we may expect strong pH gradients and hence, possible inhibition, it is important to take into account this relationship. For instance, at pH 7 it is necessary to have almost 5000 mM ammonium to attain the $K_i$ value for ammonia, whereas at pH 9

only 478 mM are needed. Therefore, for many practical situations, inhibition can be neglected for pH values below nine. Under the range of concentrations handled in this work, inhibition by ammonium will be certainly unimportant.

Nitrite has been shown to inhibit seriously *Nitrosomonas* at low pH's possibly due to the relative increase of $HNO_2$ (Hunik *et al.*, 1992, Anthonisen *et al.*, 1976, Wiesmann, 1994, Prosser, 1989). We have used the results of Hunik *et al.* (1992) as well as additional unpublished results of Meijer and Hunik to establish a quantitative relationship between the relative specific oxidation activity ($r/r_0$):

$$\frac{r_s}{r_s^0} = \frac{1.12}{(1+10^{(6.9-pH)}+10^{(pH-9.1)}} \bullet \frac{1}{(1+\frac{[NO_2^-]}{Ki_{NO_2^-}})} \qquad (5)$$

in which the $K_i$ itself depends on the pH by:

$$K_i = 0.578 \bullet \left[\frac{1}{1+10^{6.8-pH}+10^{(pH-9.0)}}\right] \bullet (1+10^{(3.3-pH)}) \qquad (6)$$

Note that these parameters were obtained by fitting the experimental data to these equations directly. Interestingly, the values pertaining the pH part of the equations were very similar to those obtained for the dependence of the specific activity and affinity constant of the pH only [Eq. (2,3)]. This corroborates the mechanistic basis of these expressions and shows that the simultaneous effects of pH and nitrite may be described by a multiplicative model of the separate effects.

Figure 2 shows the relationship between pH, nitrite concentration and relative activity. As an example (from the experimental data), for a pH of 6.5 and a in a medium containing 100 mM nitrite, the activity decreases to about 18% of that at pH 7.5 and 25 mM nitrite. The $HNO_2$ concentration (of) the former is about 40 time higher than in the latter (pKa = 3.32). These results agree with those found by Anthonisen *et al.* (1976) for *Nitrosomonas europaea.*

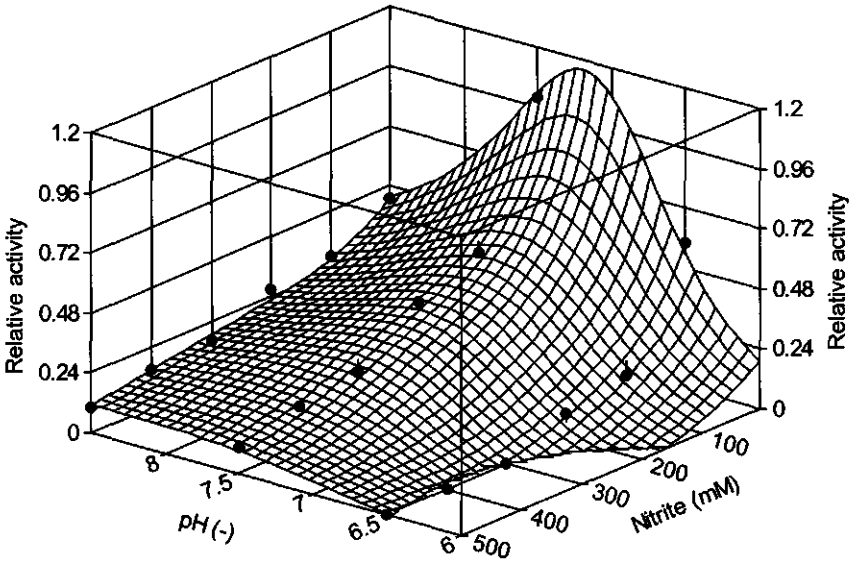


Figure 2 – Relative activity as a function of the pH and nitrite concentration as predicted by Eqs. (5,6).

*Pseudomonas* spp.

Likewise *Nitrosomonas,* nitrite has shown to inhibit denitrification specially for low pH's. Based on the measurements of Wang *et al.* (1995) Almeida *et al.* (1995) and of Thomsen *et al.* (1993), we found:

$$\frac{r_s}{r_s^0} = \frac{1.14}{1+10^{6.3-pH}+10^{pH-7.6}} \bullet \frac{1}{(1+\frac{[NO_2^-]}{Ki_{NO_2^-}})} \tag{7}$$

where $K_i$ was:

$$K_i = 0.048 \bullet \left[\frac{1}{1+10^{7.95-pH}}\right] \tag{8}$$

***Model parameters***

The dissociation constants and effective diffusion coefficients of the ions involved are shown in Table I. Strictly speaking, the diffusion rates of ions influence each other mutually (Wesseling and Krishna, 1990). However, since the exact values for each ionic species is mostly not available and since the concentration levels handled here are quite

low (activity coefficients close to unit), we considered these mutual interferences to be negligible. Consequently, it is assumed that the diffusivities of both the dissociated and undissociated forms of a given electrolyte are the same.

Table I - Model Parameters

| Parameter | Value | Dimensions | Parameter | Value | Dimensions |
|---|---|---|---|---|---|
| $\mu_{max,O2}^{Ps}$ | $2.78 \times 10^{-5}$ | $s^{-1}$ | $X^{Ps}$ | 170 | $kg.m^{-3}$ |
| $\mu_{max,NO2}^{Ps}$ | $3.33 \times 10^{-5}$ | $s^{-1}$ | $K_{l,s}^{NH4}$ | $2.21 \times 10^{-5}$ | $m.s^{-1}$ |
| $\mu_{max}^{Ns}$ | $1.52 \times 10^{-5}$ | $s^{-1}$ | $K_{l,s}^{NO2}$ | $2.21 \times 10^{-5}$ | $m.s^{-1}$ |
| $m_{O2}^{Ns}$ | $9.4 \times 10^{-4}$ | mol $O_2.kg^{-1}.s^{-1}$ | $K_{l,s}^{O2}$ | $2.63 \times 10^{-5}$ | $m.s^{-1}$ |
| $m_{NO2}^{Ps}$ | $5.44 \times 10^{-4}$ | mol $NO_2.kg^{-1}.s^{-1}$ | $K_{l,s}^{N2}$ | $2.63 \times 10^{-5}$ | $m.s^{-1}$ |
| $m_{O2}^{Ps}$ | $3.84 \times 10^{-4}$ | mol HAc.$kg^{-1}.s^{-1}$ | $K_{l,s}^{HAC}$ | $2.07 \times 10^{-5}$ | $m.s^{-1}$ |
| $Y_{NH4}^{Ns}$ | $1,66 \times 10^{-3}$ | $kg.mol^{-1}$ $NH_4$ | $D_w^{NH4}$ | $2.20 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{O2}^{Ns}$ | $1.19 \times 10^{-3}$ | $kg.mol^{-1}$ $O_2$ | $D_w^{NO2}$ | $2.20 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{O2}^{Ps}$ | $9.86 \times 10^{-3}$ | $kg.mol^{-1}$ $O_2$ | $D_w^{N2}$ | $2.83 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{NO2}^{Ps}$ | $7.39 \times 10^{-3}$ | $kg.mol^{-1}$ $NO_2$ | $D_w^{O2}$ | $2.83 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $Y_{HAC}^{Ps}$ | $1.37 \times 10^{-2}$ | $kg.mol^{-1}$ HAc* | $D_w^{HAC}$ | $2.00 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{O2}^{Ns}$ | $5.05 \times 10^{-3}$ | $mol.m^{-3}$ | $D_g^{NH4}$ | $1.78 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{NH4}^{Ns}$ | 1,25 | $mol.m^{-3}$ | $D_g^{NO2}$ | $1.78 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{O2}^{Ps}$ | $2 \times 10^{-3}$ | $mol.m^{-3}$ | $D_g^{N2}$ | $2.30 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{NO2}^{Ps}$ | 0,31 | $mol.m^{-3}$ | $D_g^{O2}$ | $2.30 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $K_{HAC}^{Ps}$ | 0,73 | $mol.m^{-3}$ | $D_g^{HAC}$ | $1.62 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $X^{Ps}$ | 36,7 | $kg.m^{-3}$ | * HAc - Acetate | | |

The thickness of the boundary layer above the gel surface was determined experimentally as described previously (Martins dos Santos *et al.*, 2001). This layer was about 50-60 microns for a stirring speed of 2000 rpm. The liquid-solid mass transfer coefficients were calculated according to the film theory on the basis of the thickness of the boundary layer measured as done before (Martins dos Santos *et al.*, 2001).

## RESULTS AND DISCUSSION

### Denitrification

Under sufficiently buffered conditions one would not expect strong pH gradients to occur within the biocatalytic support. For weak buffering, however, pH profiles are likely to develop as a result of the denitrifying activity. Figure 3 shows the measured (symbols) and predicted (lines) concentration profiles of oxygen, ammonium, nitrite and pH across a double-layered gel slab during denitrification under well (A) and poorly (B) buffered conditions. Under both buffering conditions, model predictions and experimental measurements agreed reasonably well.

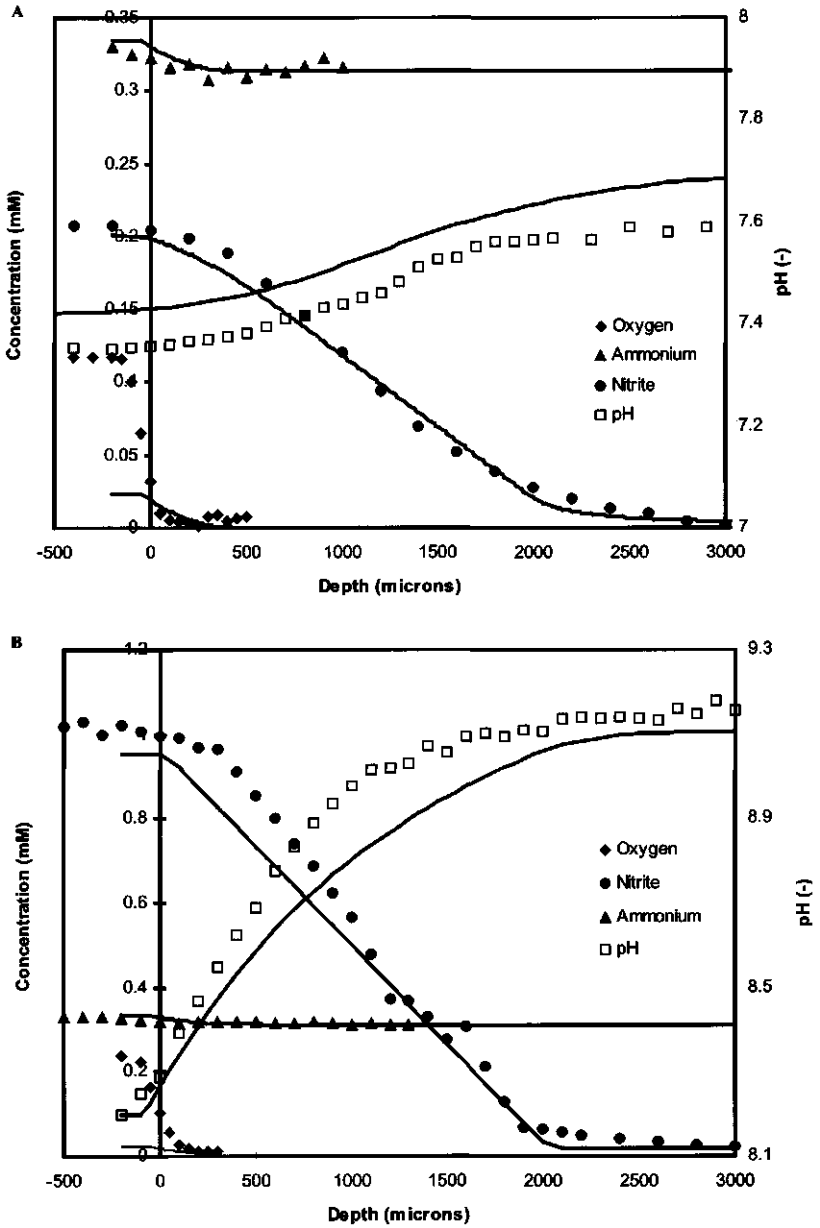


Figure 3 – Predicted (lines) and measured (points) $NH_4^+$, $NO_2^-$, pH and $O_2$ gradients across the doubled-layer gel slab under denitrifying conditions in the presence of 5 mM (A) and 0.5 mM (B) phosphate buffer. Zero represents the slab surface. From left to right: bulk liquid: -500 to – 52 μm; diffusive boundary layer: -52 to 0 μm; nitrifying layer: 0-2000 μm; denitrifying layer: beyond 2000 μm.

In both A and B a steep nitrite profile developed across the slab, whereas the ammonium concentration remained practically at the same level. Due to the low oxygen concentration, there was only a little nitrification within the very first outer layers. Nitrite, on the contrary, was consumed at a considerable rate within the first layers of the denitrifying zone. The shape of the nitrite profile reflects its diffusion across the nitrifying zone, being driven by the concentration gradient between the bulk and the denitrifying layer, which virtually works as a "sink". From the Figure it seems also clear that the *Pseudomonas* sp. within the first layers of the denitrifying zone would have accounted for most of the denitrification. The actual oxygen profiles were sharper than predicted likely, as reported earlier (Martins dos Santos *et al*, 2001), due to its uptake at the slab surface by undefined heterotrophic populations. The oxygen level in the bulk (about 0.1 mM) in both A and B was higher than predicted because it was difficult to maintain a constant (low) oxygen tension in such an open vessel. In any case, these discrepancies did not affect the denitrification process as a whole.

Although the general trend in ammonium, oxygen and nitrite profiles was similar under both buffering conditions the shape of the respective pH profiles differed considerably. Under relatively well-buffered conditions (A), the pH increased only about 0.2 units across the whole slab, whereas for the lower buffering capacity (B) this increase amounted more than one pH unit. This difference was due to the weaker capacity of the buffer in B, which was unable to compensate for the alkalinity generated by reduction of nitrite to nitrogen cf. Eq (2). From the profiles measured, however, it was unclear whether the pH increase did indeed result in a lower nitrite reduction rate, as predicted by Eq. (6). Nevertheless, and despite the simplifications done, the model was able to account for the interactions among all the charged species with a reasonable accuracy under the conditions tested. These results confirm those predicted long ago by Riemer & Harremoes (1978) and measured indirectly by Arvin & Kristensen (1982).

**Nitrification**

Figure 4 shows the predicted (lines) and measured (symbols) oxygen, ammonium, nitrite and pH profiles across the gel slab under purely nitrifying conditions (i.e., with no organic carbon supplied) for a well (A) and poorly (B) buffered influent.

In both A and B, ammonium was consumed at about the stoichiometric rate of nitrite accumulation. All ammonium that was consumed was thus transformed into nitrite. The general shape of the curves (regardless of their absolute values) indicates that nitrifiers were active throughout the whole 2000-μm layer and that nitrite was not further converted into nitrogen through denitrification.

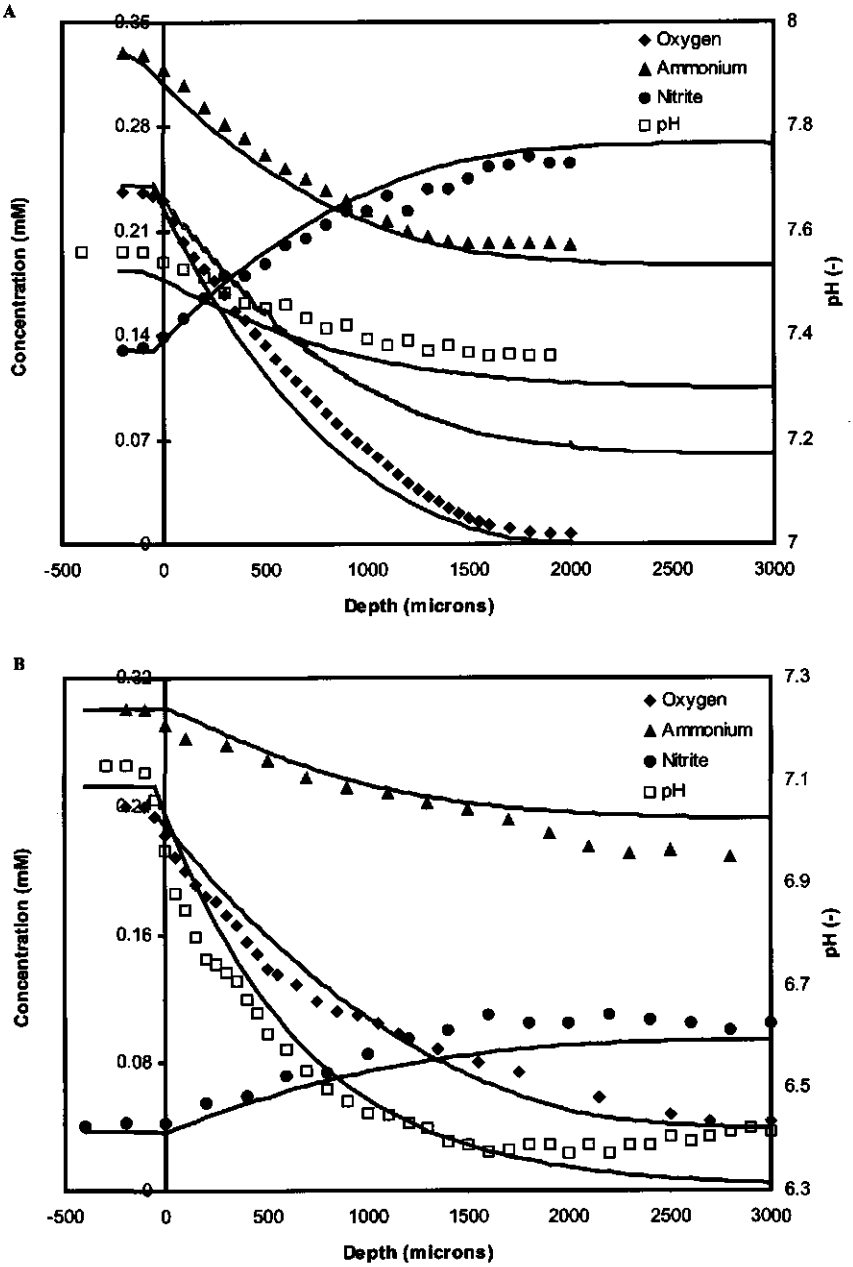


Figure 4 – Predicted (lines) and measured (points) $NH_4^+$, $NO_2^-$, pH and $O_2$ gradients across the doubled-layer gel slab nitrifying conditions in the presence of 5 mM (A) and 0.5 mM (B) phosphate buffer.

Note also that, as shown earlier (Martins dos Santos *et al.*, 2001), the predictions for oxygen took into account heterotrophic oxygen uptake by the denitrifying bacteria in the inner slab. In well buffered medium (4A), the pH profile was relatively smooth (pH difference of about 0.2 units), whereas under poorly buffered (4B) conditions a steep pH gradient of about 1 pH unit developed across the slab. The gradient was far more pronounced in the nitrifying slab, after which it seemed to increase slightly. Conversely, the oxygen, ammonium and nitrite profiles were much less steep in the poorly buffered medium than for that well buffered. In fact, the ammonium consumed and ammonium produced in B were less than half of that in A. Likely, the reduced nitrification in B resulted from the inhibitory effect of the pH decrease that arose from the nitrifying activity itself (cf. Eq. (2)). Evidently, the buffer capacity of medium B was not sufficient to compensate for the acid production. Globally, this reflected as well on a lower bulk pH (7.1) than that of the influent (7.5).

These effects were correctly described by the model, which predicted fairly well a reduced nitrifying activity as a result of a pH decrease. Also, not only the oxygen profile in B was less steep than in A, but also oxygen was not completely depleted even after 3000 microns. This likely means that the pH inhibited as well the heterotrophic activity of the denitrifiers in the denitrifying slab. The model accounted for this as well. The steepness of the pH profile across the slab in the poorly buffered medium also clearly illustrates the importance of external resistance to mass transfer on the development of substrate and product gradients and on the kinetics of nitrogen conversion. Indeed, although the system was well stirred and the thickness of the diffusive boundary layer was estimated to be only about 50 microns (Martins dos Santos *et al.*, 2001), within this layer the pH dropped between 0.2 and 0.3 pH units. External mass transfer resistance influences the in/efflux of acids and bases into / out-of a catalytic particle and therefore, in weakly buffered systems, internal pH values may be suboptimal despite of influent pH corrections. These results as a whole generally agree with those presented earlier by De Beer *et al.* (1993) and Zhang & Bishop (1995, 1996), who measured directly pH profiles in biofilms and with those of Szwerinski *et al.* (1986) and Siegrist & Gujer (1987), who predicted and estimated indirectly such gradients.

**Simultaneous nitrification and denitrification**

One of the advantages of coupling nitrification and denitrification within a single biocatalytic particle is, as claimed by several authors (Kokufuta *et al.*, 1987, 1988 ,Kurosawa & Tanaka, 1990, Martins dos Santos *et al.*, 1996, Tartakovsky *et al.*, 1996), the possibility of partially compensating the acid produced in nitrification (Eq. (1)) by

the base formed in the denitrifying step (Eq. (2)). pH control would be thereby greatly facilitated, which is particularly relevant for wastewater treatment at large scales. Hence, if that is indeed so, one would expect that the pH gradient throughout a biocatalyst support such as that studied here would be a "combination" of the profiles of Figures 2 and 3 above. Figure 4 shows the oxygen, ammonium, nitrite and pH profiles across the double-layered slab under well (A) and poorly (B) buffered conditions for coupled nitrification and denitrification. As expected, at higher buffer capacity (4A), the pH profile across the slab was fairly flat, with only a slight "bow" (lower than 0.1 pH unit) within the nitrifying zone, and was followed by a small increase thereafter. This profile (symbols) was actually flatter than that predicted by the model (line) for both the nitrifying and denitrifying zones. The lowest pH value corresponded to the point where (by taking into account mass transfer and the influence of all other ionic species) the acid and base resulting from nitrification and denitrification compensated each other. The corresponding experimental oxygen, ammonium and nitrite profiles agreed fairly well with the model predictions and reflected a relatively high nitrogen transformation rate (see inset in 4A).

For lower buffer capacity (4B), there was a slight pH decrease within the first 500 microns and a progressive increase in the following 2000. However, these changes were less pronounced than those anticipated by the model, which had predicted a maximal pH drop of about 0.2 within the first 100 microns and an increase of about 0.4 units thereafter. Thus, in fact, the system seemed to have a buffer capacity higher than that predicted, even by taking into account that nitrogen removal activity was slightly lower than foreseen (as indicated by the measured ammonium and nitrite profiles). The slight deviation between the predicted and measured nitrogen removal activities is likely to be explained by the somewhat higher-than-predicted oxygen consumption at the bead surface by undefined heterotrophic populations. Indeed, we could observe a very thin layer of cells covering the slab surface after supplying continuously acetate to the reactor (to allow denitrification to occur) for a longer period of time. Likely, these cells were taking up some of the oxygen in the bulk for acetate oxidation. This has been observed earlier in both nitrifying (van Benthum *et al.* 1996) and nitrifying /denitrifying systems (Martins dos Santos *et al.*, 1996, 2001).

A careful comparison between Figure 4B (poorly buffered nitrification) and 5B (poorly buffered nitrification and denitrification) shows that both the ammonium oxidation rate and its extent were higher in the co-immobilized system than in that with nitrifiers alone.

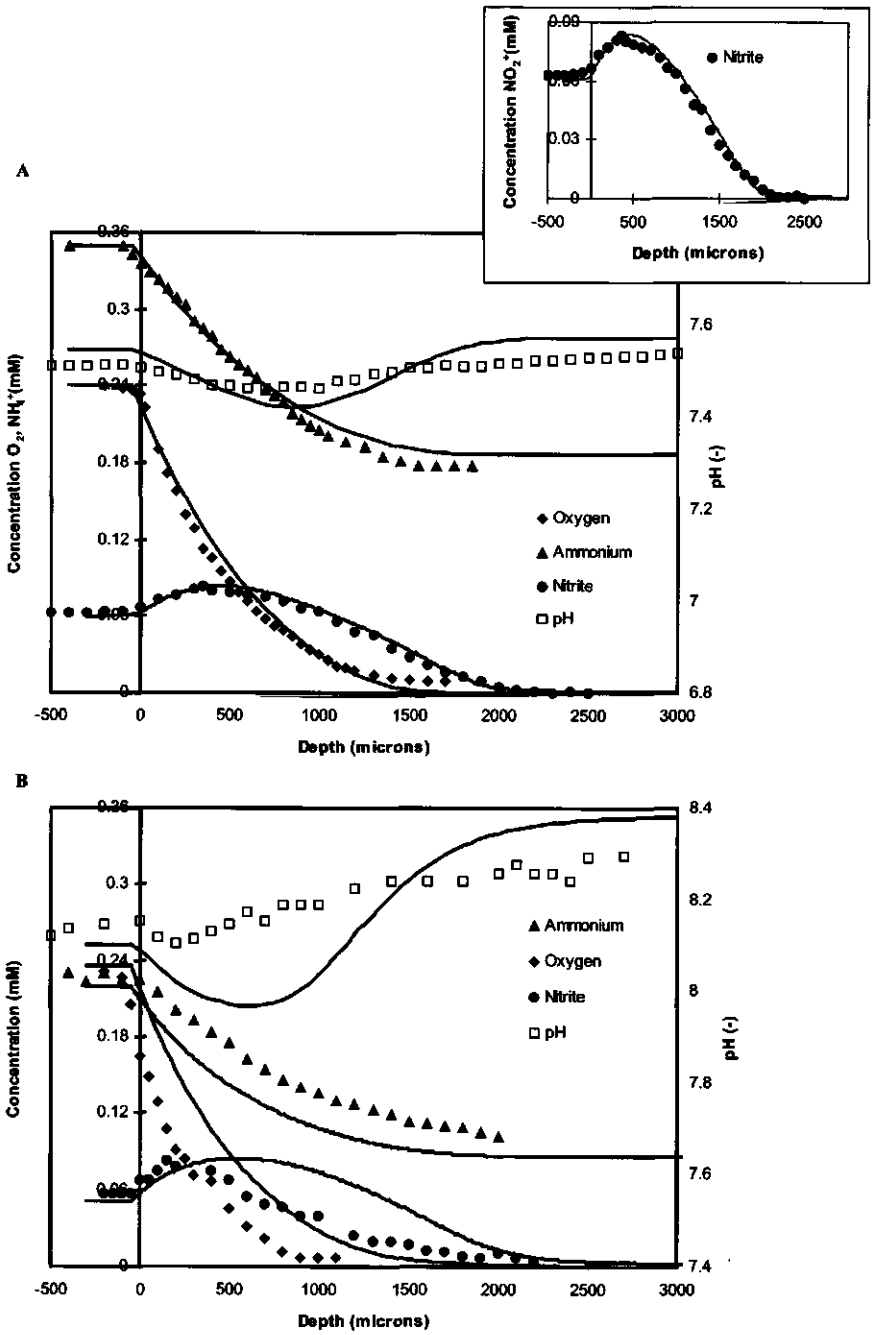


Figure 5 - Predicted (lines) and measured (points) $NH_4^+$, $NO_2^-$, pH and $O_2$ gradients across the doubled-layer gel slab under coupled nitrifying and denitrifying conditions in the presence of 5 mM (A) and 0.5 mM (B) phosphate buffer.

Indeed, although the ammonium oxidation under poorly buffered conditions was about two thirds of that under good buffering, this rate was still almost twice as high as that for nitrification alone in weakly buffered medium (Figure 4B). Also, in 5B there is barely any oxygen beyond 2000 microns, which indicates a higher oxygen consumption activity both by the nitrifiers and denitrifiers. Both this enhanced ammonium oxidation and the higher oxygen uptake with regard to unbuffered nitrification are likely to be explained by the denitrifying activity in the inner slab, in which the denitrifiers partly counteract the acidity produced in the nitrifying step. These results confirm experimentally the qualitative predictions made earlier by Kokufuta *et al.* (1987, 1988), Martins dos Santos *et al.* (1996) and Tartakovski *et al.* (1996).Although advantageous with regard to pH control, it was not entirely clear why the system in Figure 5B seemed to have a buffer capacity higher than predicted. One plausible explanation is simply that the interactions among the various ionic species involved were not correctly handled by the model, which is actually very likely given their complexity (such as that regarding the role of the carbonate / bicarbonate pair, which is a particularly "difficult" pair) and the simplifications made here (see Model development above). Discrepancies in the pH evolution in methanogenic and nitrifying microbial aggregates observed by other authors were partly explained by the buffer capacity of the biomass itself (which can be quite significant, as shown by De Beer *et al.*, 1993), and by processes related to cell lysis or ammonification of proteins (Lens *et al.*, 1993). Neither of these effects, however, is likely to have been important here because the biomass concentrations involved were low and the experiments did not last long enough so as to expect significant cell lysis.

**Influence of the external pH, buffer capacity and bulk $NH_4^+$ concentration**

Given the close interrelatedness of the underlying processes, it is difficult to assess the contribution of each of the factors involved on the final pH development throughout a nitrifying / denitrifying particle. During nitrification, ammonium, oxygen and bicarbonate are consumed and must diffuse from the bulk into the support. Nitrite and carbon dioxide are produced and diffuse both outwards and inwards. In the denitrifying step, acetate should diffuse inwards and is consumed together with the nitrite produced. Concomitantly, nitrogen and bicarbonate are produced, which then diffuse outwards. The pH at each point determines the ratio of ionized to unionized form of each species. The concentration of each ionized species contributes to the ionic strength of the medium, which ultimately will influence its total buffering capacitym wchich in turn is strongly dependent of the concentration of the "buffering" ionic pairs. To gain some

understanding of the interplay of these factors, we studied the influence of the influent bulk pH, of the bulk capacity of the medium and of the concentration of ammonium on the development of pH profiles.

***External pH***

Figure 6 shows both predicted and measured of pH gradients across the slab under nitrifying conditions for several initial pH values in a weakly (0.5 mM phosphate) medium. There is a clear influence of the bulk pH on the pH gradients that develop across the slab. Interestingly enough, the pH drop is larger for higher bulk pH's than for lower ones, particularly for those under 6.5. This is a direct consequence of the dependence of the nitrifying activity (both $K_s$ and $r_s$) on the pH as expressed in Eq.(3). According to this dependence, nitrifying cells are a lot more inhibited by lower than by higher pH values. Therefore, at low pH's ammonium conversion is low and less acidity is produced, which then results in a less dramatic decrease of pH as compared to higher pH's.

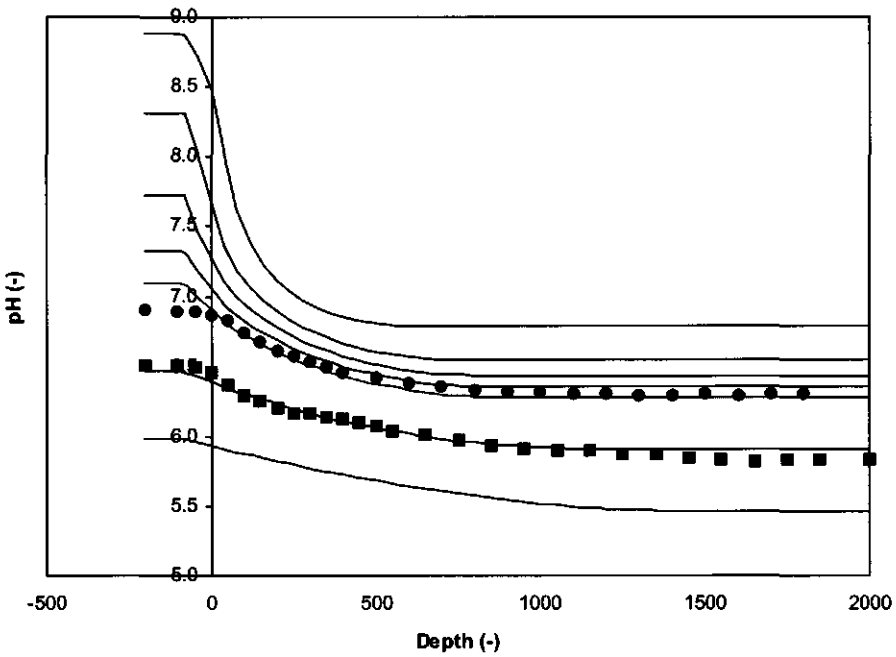


Figure 6 – Effect of the bulk pH on the pH gradient across the slab under nitrifying conditions (0.25 mM phosphate buffer). Symbols: measurements; lines: predictions.

A reverse picture is produced when considering denitrification. Indeed, denitrifying cells are somewhat less sensitive to low pH's than nitrifiers and therefore the pH across the slab increases more at low bulk pH than at higher ones, as a result of the larger acid-base decompensation (not shown). Such behavior has been earlier described by Riemer & Harremöes (1978) and Arvin & Kristensen (1982).

The combination of both pictures yields one (see also Figure 8), in which the predicted pH profiles are plotted for several values of bulk pH. Accordingly, at higher pH values, the pH drop within the nitrifying layer is a lot larger than at lower pH's. Conversely, the pH increase in the inner part as a result of denitrification is larger at lower pH values. In any case, at both low and high pH the gradients developed are a lot less pronounced than those for nitrification or denitrification only, which clearly illustrates the advantage of combining these two processes in the same biocatalyst particle. The ammonium, nitrite and oxygen gradients developed across the slab under the different conditions mirror the pH profiles shown in Figure 6, which therefore represent the overall outcome of such study. Hence, we do not show here these gradients.

***Effect of the buffer capacity***

The above analysis of the influence of the external pH on the nitrogen removal activity (and, consequently, on the development of pH gradients) across the slab has been made for a given, constant, buffer capacity of the influent medium. The effects described, however, depend strongly on the buffer capacity of the medium as well. Szwerinski and co-workers (1986) have shown experimentally that pH drops across a biofilm were correlated to the total alkalinity of the medium at a certain medium pH. Based on mass transport relations and on chemical equilibrium they predicted the development of increasingly steeper pH gradients across a nitrifying biofilm for decreasing alkalinity. For low alkalinities, however, their predictions were far too dramatic and the observed pH drops were much lower than foreseen, as experimentally demonstrated by Zhang *et al.* (1996). In Figure 7 the predictions of the model developed here adapted for a biofilm are plotted together with the pH profiles measured by Zhang *et al.* (1996) at different alkalinity levels.

Clearly, the lower the alkalinity the greater was the pH gradient. Beyond a certain point, however the profiles leveled off to a pH of about 5.4. Indeed, for such pH values the nitrifying activity is strongly inhibited (cf. Eq 5) and ammonium oxidation is practically arrested. Therefore, the pH did not decrease further. The previous model of Szwerinski *et al.* (1986) did not take into account pH inhibition. As pointed out by Szwerinski, "the interesting point is that pH-inhibition and reduced nitrogen removal due to this can

occur without the effect being detected in the bulk medium". Note as well that these effects arose already within the first 300 microns.

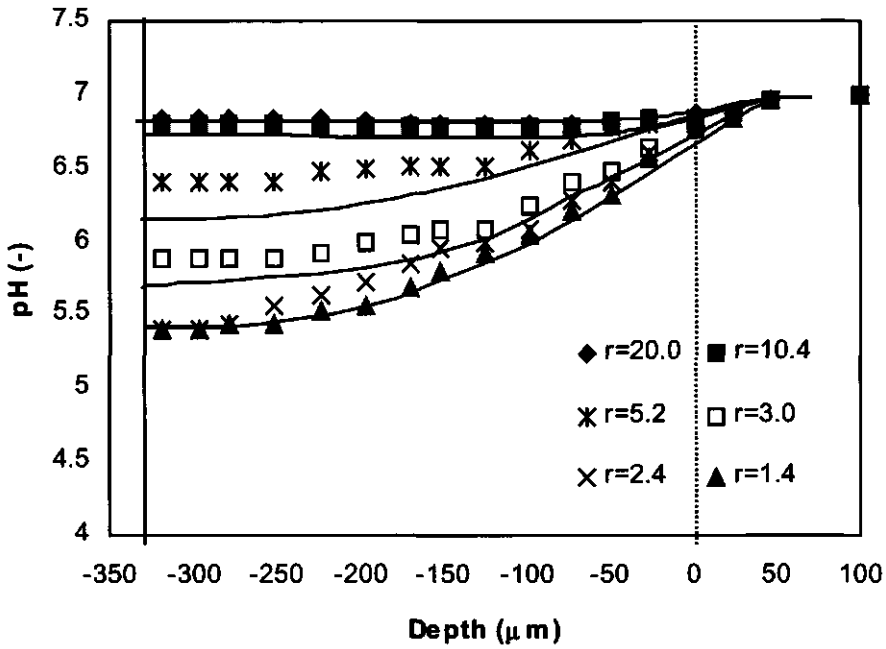


Figure 7 – Experimental (symbols) and predicted (lines) pH profiles across a fully developed nitrifying biofilm for different alkalinities (ratio $HCO_3/O_2$) and a medium pH of 7. Experimental data operating parameters and picture format as described in Zhang *et al.* (1996). Dashed line represents biofilm-bulk interface; solid vertical line represents end on nitrifying zone.

For denitrification, similar considerations hold. For decreasing alkalinity the pH gradients increase up to a point where the denitrifying activity is completely inhibited and no further gradients develop. These issues have been earlier described by Arvin & Kristensen (1982) and are further discussed below (see Figure 8).

**Combined effects**

Under all conditions tested here, the model described appropriately the pH gradients developed, as well as the corresponding substrate and product profiles across the slab (Figures 2 to 5).

established. Therefore, we used the model to construct plots that account for the combined effects of pH and buffer capacity (Figure 9).

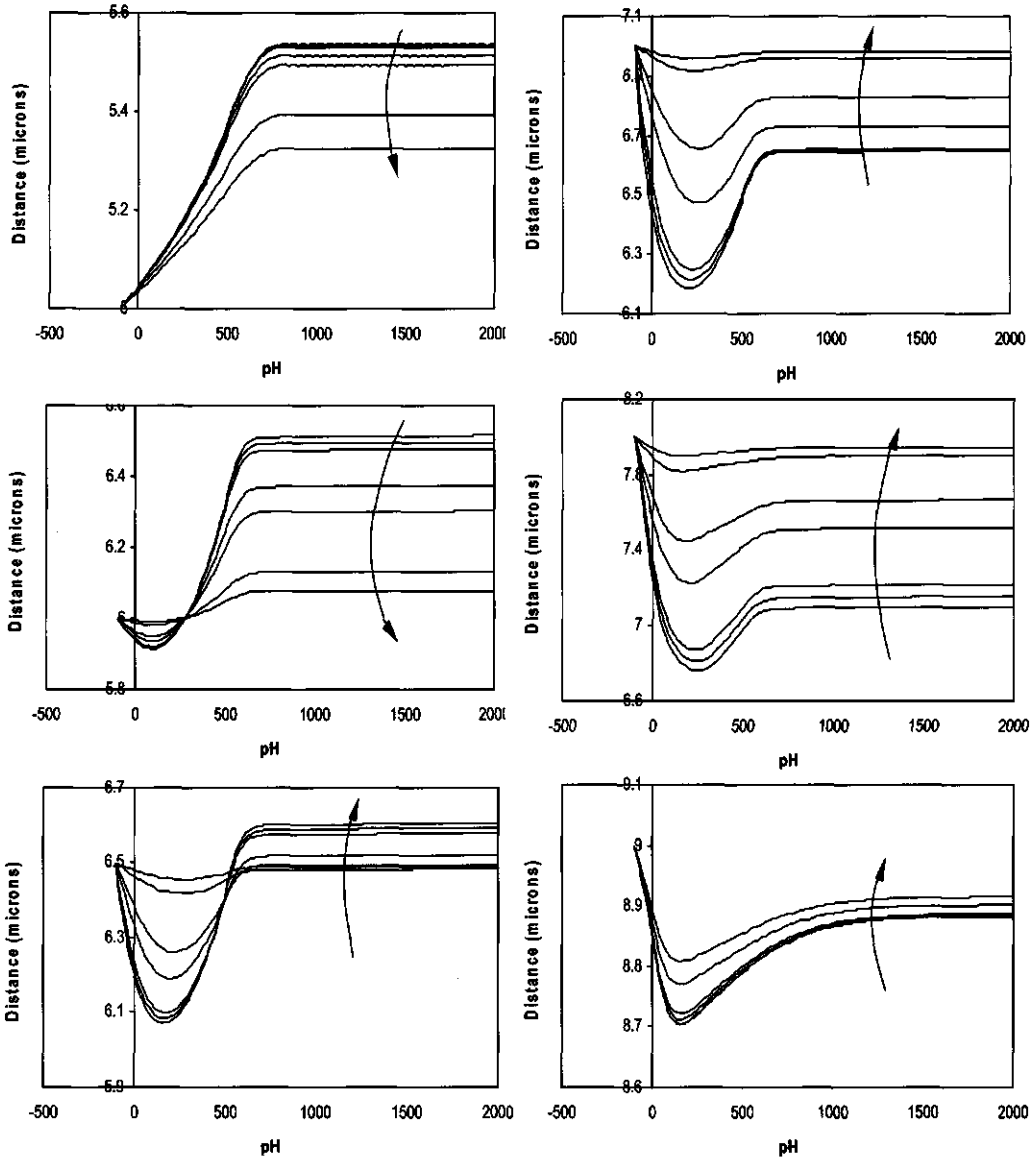


Figure 9 – Effect of the buffer capacity and pH of the medium on the pH gradients across the slab for simultaneous nitrification and denitrification. The arrows indicate increase in buffer capacity for phosphate buffer (which were: 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 mM). Note the difference in scale.

Also, it described well pH gradients measured independently by others (Zhang et al. 1996). The validity of the model under these conditions has been thereby established. Therefore, we used the model to construct plots that account for the combined effects of pH and buffer capacity (Figure 9).

Clearly, at both high and low pH's the gradients (either drops or rises) are smaller than at intermediate pH, which reflects the inhibition of microbial activity under more extreme conditions. Inhibition, however, has both a direction and a different magnitude for nitrifiers and denitrifiers. At lower pH, nitrifiers ppear to be more strongly inhibited than denitrifiers and at higher pH the reverse is true, although to different extents. This reflects not only intrinsic inhibition by pH (as excess of $HNO_2$ or $H^+$ ions, for instance) but also the decreased affinity of nitrifiers for ammonia at lower pH and that of denitrifiers for nitrite at higher pH (see Eqs.(4) and (7) respectively. At pH 6.5 the general shape of the profiles in reversed with regard to the effect of buffer capacity (note the reversion of the direction of the arrows).

As the pH gradients are less pronounced at extreme pH's, the effect of buffer capacity is also, in relative terms, less important than at neutral values. Indeed, for pH between 6.5 and 8 (within the optimal pH range for both bacteria) the buffer capacity of the medium determines the magnitude of the pH gradients. At pH 8, for instance, the pH drop ranges from almost 1.5 pH unit at a phosphate concentration of 0.01 mM to practically 0 for phosphate at 10 mM. At this pH and with adequate buffering (as low as 5 mM phosphate, which is currently considered a low buffering capacity), acidification and alkalinisation virtually compensate each other and the degrading activity of both nitrifiers and denitrifiers is maximised. For nitrification or denitrification only, higher buffer capacities are required to maintain microbial activity.

***Effect of $NH_4^+$***

Under the conditions described for the experiments above, the contribution of the substrates ammonium and acetate to the total buffer capacity of the system was relatively low because their concentration in medium was low as well. For poorly buffered systems, the contribution of these substrates to the total buffer capacity of the medium may be important if they are present at higher concentrations. Indeed, both the ammonium ion and acetate, though weak acids, are likely to influence the total buffer capacity of the system if this is low enough. To test this hypothesis, we measured pH profiles across a fully active nitrifying taken out of a fluidised bed (as described in De Beer *et al.*, 1993) and supplied to the medium with 1 and 10 mM ammonium (Figure 10) and 0.25 mM phosphate. The experiments were carried out under oxygen-limiting

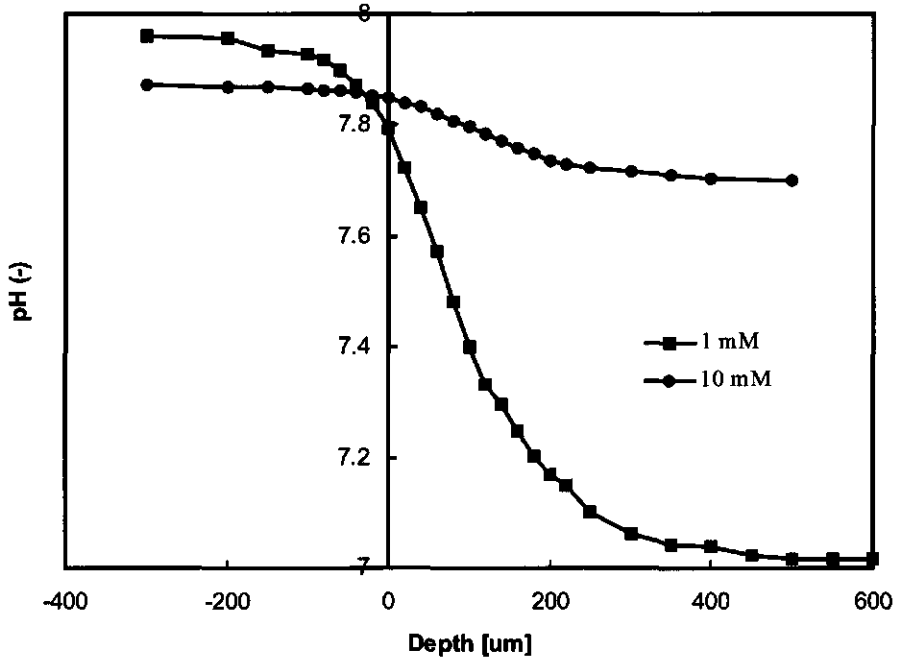


Figure 10 – Effect of the bulk concentration of ammonium on the pH gradients across the slab under nitrifying conditions for fully developed nitrifying aggregates (0.5 mM phosphate buffer). Symbols: measurements; lines: predictions.

## FINAL REMARKS

In this work, we described both a conceptual framework and a detailed experimental analysis of the pH effects on coupled nitrification and denitrification. The results obtained and the data generated have clearly shown that coupling nitrification and denitrification within a single catalytic particle is an effective means of minimizing inhibition of microbial activity by pH. Due to the partial compensation of acidification resulting from nitrifying activity by alkalinisation due to denitrifiers, a much more robust multistep process is attained. This has great significance for the practice of waste and industrial water treatment and greatly facilitates pH control, which requires much more buffer capacity than for conventional systems. Hence, information on the specific sensitivity of nitrifiers or denitrifiers to pH, together with the knowledge of the influence of different factors on pH development (buffer capacity, bulk pH and so forth) as well as the time scale for pH development yield, as a whole, a broad framework for predicting and handling pH effects on the performance of nitrogen removal systems.

sensitivity of nitrifiers or denitrifiers to pH, together with the knowledge of the influence of different factors on pH development (buffer capacity, bulk pH and so forth) as well as the time scale for pH development yield, as a whole, a broad framework for predicting and handling pH effects on the performance of nitrogen removal systems.

**ACKNOWLEDGEMENTS**

We acknowledge the financial support given by the Junta Nacional de Investigação Científica e Tecnológica (JNICT), Portugal.

**NOTATION**

| | | |
|---|---|---|
| $D$ | dilution rate of the reactor | [$s^{-1}$] |
| $I$ | Component | [-] |
| $m$ | Microorganism | [-] |
| $\mathbf{D}_{gel}^{i}$ | diffusion coefficient component i in the gel | [$m^2.s^{-1}$] |
| $\mathbf{D}_{w}^{i}$ | diffusion coefficient component i in water | [$m^2.s^{-1}$] |
| $k_{l,g}^{i}$ | gas-liquid mass-transfer coefficient component i | [$m.s^{-1}$] |
| $k_{l,s}^{i}$ | liquid-solid mass-transfer coefficient component i | [$m.s^{-1}$] |
| $K^{m}_{i}$ | monod constant *m* for I | [$mol.m^{-3}$] |
| $m_i^m$ | maintenance *m* on I | [mol. $kg^{-1}$ $s^{-1}$] |
| $R$ | radius to the centre of the bead | [m] |
| $R_{max}$ | maximum radius of the gel bead | [m] |
| $R_s$ | substrate consumption rate | [$mol.m^3.s^{-1}$] |
| $R_x$ | growth of the bacteria | [$kg.m^{-3}$.s-1] |
| $R_s^{sus}$ | substrate production by suspended bacteria | [$mol.m^{-3}.s^{-1}$] |
| $R_x^{sus}$ | production of suspended biomass | [$kg.m^{-3}.s^{-1}$] |
| $S$ | substrate concentration | [$mol.m^{-3}$] |
| $S'$ | limiting substrate concentration | [$mol.m^{-3}$] |
| $S_{bulk}$ | substrate concentration in the bulk phase | [$mol.m^{-3}$] |
| $X$ | concentration of biomass | [$kg.m^{-3}$] |
| $X^{m}_{bulk}$ | concentration of suspended biomass *(m)* | [$kg.m^{-3}$] |
| $X^{m}_{max}$ | max. biomass conc. in the gel microorganism (m) | [kg.m-3] |
| $Y^{m}_{i}$ | yield microorganinsm (m) on substrate (i) | [kg/mol i] |
| $\mu^{m,i}_{max}$ | maximum growth rate of (m) on (i) | [$s^{-1}$] |

## REFERENCES

Almeida,J.S., Reis, M.A.M., Carrondo, M.J.T. (1995). Competition between nitrate and nitrite reduction in denitrification by *Pseudomonas fluorescens*. Biotech. & Bioeng. 46:476-484.

Anthonissen, A.C., Loehr, R.C., Prakasmam, T.B.S., Srinath, E.G. Inhibitionof nitrification by ammonia and nitrous acid. 1976. Journal WPCF, 48:835-852

Antoniou, P., Hamilton, J., Koopman, B., Jain, R., Holloway, B., Lyberatos, Svoronos, S.A. 1990. Effect of temperature and pH on the effective maximum specific growth rate of nitrifying bacteria. Wat. Res.24:97-101.

Arvin, E., Kristensen, G.H. 1982. Effect of denitrification on the pH of biofilms. Wat. Sci. Technol. 14:833-848

De Beer, D. and van den Heuvel, JC. Response of ammonium-selective microelectrodes based on the neutral carrier nonactin, Talanta, 35 (9) 728-730 (1988).

de Beer, D., Huisman, J.W., Van den Heuvel, J.C., Ottengraf, S.P.P. 1992 The effects of pH profiles in methanogenic aggregates on the kinetics of acetate conversion. Wat. Res. 26:1329-1336

de Beer, D., Van den Heuvel, J.C. Ottengraf, S.P.P. 1993. Microelectrode measurements of activity distributions in nitrifying bacterial aggregates. Appl. Environm. Microbiol. 59:573-579

Cronenberg CCH. 1994. Biofilms investigated with needle-type glucose sensors. PhD dissertation University of Amsterdam, Amsterdam, The Netherlands

Dombrowski T. 1991. Kinetik der Nitrifikation und Reaktionstechnik der Stickstoffeliminierung aus hochbelasteten Abwässern. VDI-Fortschrittsberichte, Reihe 15:Umwelttechnik nr 87

Flora, J.RV., Suidan, M.T., Biswas, P., Sayles, G.D.1993. Modeling substrate transport into biofilms: role of multiple ions and pH effects. J. Environm. Eng. 119:908-930

Flora, J.RV., Suidan, M.T., Biswas, P., Sayles, G.D.1995. A modeling study of ananerobic biofilm systems: I. Detailed biofilm modeling. Biotechnol Bioeng. 46:43-53

Heijnen JJ, van Loosdrecht MCM, Mulder R, Weltevrede R, Mulder A. Large scale anaerobic-aerobic treatment of complex industrial wastewater using biofilm reactors. Wat. Sci. Technol. 1991; 23:1427-1436

Hunik J.H. Tramper J., Meijer H.J.G. (1992). Kinetics of *Nitrosomonas europaea* at extreme substrate, product and salt concentrations. Appl. Microb. and Biotechnol. 37:802-807.

Hunik J.H., Bos C.G., Van Den Hoogen P., De Gooijer C.D., Tramper J. (1994). Co-immobilized *Nitrosomonas euroapaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in k-carrageenan gel beads. Biotechnol. Bioeng. , 43:1153-1163.

Kurosawa H, Tanaka H (1990) Advances in immobilized cell culture: development of a co-immobilized mixed culture system of aerobic and anaerobic micro-organisms. Process Biochem Int 25:189-196

Lens PNL, de Beer D, Cronenberg CCH, Howen FP, Ottengraf SPP, Verstraete WH. 1993. Heterogeneous distribution of methanogenic activity in UASB aggregates. Appl. Environm. Microbiol. 59:3803-3815.

Mankad, T. and Nauman, E.B. (1992). Modeling of microbial growth under dual limitations. The Chemical Engineering Journal 48:B9-B11

Martins dos Santos V.A., Tramper J., Wijffels R.H. (1993). Simultaneous nitrification and denitrification using immobilized micro-organisms. Biomat., Art. Cells & Immob. Biotech., 21(3),317-322.

Martins dos Santos, V.A.P., L. Marchal, Tramper, J., R.H. Wijffels. (1996). Modelling and evaluation of an integrated nitrogen removal system with co-immobilized microorganisms. Biotechnol. Prog. 12:163-171

Martins dos Santos, V.A.P., Tramper, J., R.H. Wijffels. (1996) The Magic Bead Concept: An Integrated Approach for Nitrogen Removal with Co-immobilized Microorganisms. Appl. Microbiol. Biotechnol. 45:447-453.

Martins dos Santos, V.A.P., Verschuren, P., van den Heuvel, H., Tramper, J., Wijffels, R. (2001). Substrate and product profiles across double-layer gel beads: modelling and experimental evaluation. Biotechnol Bioeng, Submitted

Picioreanu C, van Loosdrecht MCM, Heijnen JJ. (1998a) A new combined differential discrete cellular automaton approach for biofilm modelling: Application to growth in gel beads. Biotech. Bioeng 57: 718-731

Picioreanu C, van Loosdrecht MCM, Heijnen JJ. (1998b) Mathematical modeling of biofilm structure with a hybrid differential-discrete approach. Biotech. Bioeng 58: 101-116

Prosser JI. Autotrophic nitrification in bacteria. 1989. Adv. Microbial Physiol. 30:125-181

Riemer, M., Harremoes, P. 1978. Multi-component diffusion in denitrifying biofilms. Prog. Wat. Technol. (presently Wat. Sci. Technol.) 10(5/6):149-163

Sheintuch, M., Tartakovski, B., Narkis, N., Rebhun, M.1995. Substrate inhibition in a continuous nitrification process. Wat. Res. 29:953-963

Siegrist, H., Gujer, W. 1987. Demonstration of mass transfer and pH effects in a nitrifying biofilm. Wat. Res. 21:1481-1487

Suzuki, I., Dular, U., Kwok, S.C. Ammonia or ammonium as substrate for oxidation by *Nitrosomonas europaea* cells and extracts. J. Bacteriol. 1974, 120:556-558

Szwerinski, H., Arvin, E., Harremoes, H. 1986. pH-decrease in nitrifying biofilms. Wat. Res. 20:971-976.

Tan Y, Wang Z-X, Marshall KC. 1998. Modeling pH effects on microbial growth: a statistical thermodynamic approach. Biotechnol. Bioeng. 59:724-731

Tan Y, Wang Z-X, Marshall KC. 1996. Modeling substrate inhibition of microbial growth. Biotechnol. Bioeng. 52:602-608

Tanaka K, Sumino T, Nakamura H, Ogasawara T, Emori H. Application of nitrification by cells immobilized in polyethylene glycol. In: Immobilized cells: basics and applications (Wijjfels RH, Buitelaar, RM, Bucke, C Tramper, J, Eds). Elsevier Science, The Netherlands, 1996, pp. 718-724

Tijhuis L. The biofilm airlift suspension reactor: biofilm formation, detachment and heterogeneity. 1994, PhD thesis, Delft University of Technology, Delft, The Netherlands.

Thomsen, J.K., Geest, T., Cox, R.P. 1994. Mass spectrometric sudies of the effect of pH on the accumulation of intermediates in denitrification by *Paracoccus denitrificans*. Appl. Environm. Microbiol. 60:536-541

Van Benthum WAJ, van Loosdrecht MCM, Heijnen JJ. Nitrogen removal using nitrifying biofilms and denitrifying suspended growth in a biofilm air-lift suspension reactor. Proceedings of the 3rd International IAQW special conference on biofilm systems, Copenhagen, 27-30 August 1996

Van Loosdrecht MCM, Heijnen JJ. Bioreactors for wastewater treatment. Trends in Biotechnol. 1993; 11:117-121

Wang, J.H., Baltzis, Lewandowski. (1995) Fundamental denitrification studies with *Pseudomonas denitrificans*. Biotech. & Bioeng., 47:26-41.

Wiesmann, U. Biological nitrogen removal from wastewaters. In: Fletcher, A. (De.): Advances in Biochemical Engineering and Biotechnology. Springer-Verlag, 1994, 51:113-154

Wijffels RH, Eekhof MR, Van den Heuvel, JC, Tramper J. Pseudo-steady-state oxygen concentration profiles in ana agar slab containig growing *Nitrobacter agilis*. J. Ferment. Bioeng. 1995, 2, 167-170

Wijffels R.H., De Gooijer C.D., Kortekaas S., Tramper J. Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in k-carrageenan. Part 2: Model evaluation. *Biotechnol. Bioeng. 1991*, **38**: 232-240

Willaert RG, Baron GV, De Backer L. Immobilized living cell systems. 1996, John Wiley & Sons, New York, USA

Zhang TC, Bishop PL. 1996. Evaluation of substrate and pH effects in a nitrifying biofiul,. Water Environm. Res. 68:1107.1115

This chapter has been submitted for publication by Martins dos Santos VAP, Jacobs M, Jetten MSM, Kuenen JG, Tramper J, Wijffels RH.

# CHAPTER 10

# Simultaneous Autotrophic Nitrification and Anaerobic Ammonium Oxidation with Co-immobilized Micro-organisms

## ABSTRACT

A compact, fully autotrophic single-staged system is proposed to combine "classical" aerobic nitrification by autotrophic nitrifying cells with the recently discovered anaerobic ammonium oxidation process (Anammox). The system is based on the "magic-bead" concept, in which nitrifying and anaerobic ammonium-oxidizing microorganisms are co-immobilized in double-layer gel beads. In this combined process, the ammonium supplied to the reactor medium is partly oxidized by nitrifying cells immobilized in the outer layer of the beads. Due to the fast oxygen uptake by the nitrifiers, both biomass and substrate profiles develop leading to the establishment of extensive anoxic zones within the beads. The nitrite produced by the aerobic nitrifiers and the remaining ammonium are then anaerobically converted into nitrogen by the Anammox microorganisms entrapped in the bead core. This system was shown to work both in batch and in continuous mode. Head-space analysis showed that the Anammox sludge was solely responsible for nitrogen formation. Overall nitrogen removal rates up to 1.5 kg-N.$m^{-3}$.$day^{-1}$ were achieved in continuously operated air-lift loop reactors (15 mM $NH_4^+$ in feed, 20% v/v beads, 4-5 mm bead diameter). These results show that the proposed system may constitute a valuable alternative for effective nitrogen removal from wastewaters. To our knowledge, this is the first report of a single-staged, fully autotrophic system capable of converting ammonia to dinitrogen gas without the need of an external electron acceptor other than oxygen.

## INTRODUCTION

Nitrogen compounds are commonly removed from wastewater by a combination of two processes: aerobic autotrophic nitrification and anaerobic heterotrophic denitrification. In nitrification, ammonium is oxidized first to nitrite and subsequently to nitrate by autotrophic nitrifying cells with oxygen as electron acceptor. The nitrate produced is reduced (via nitrite) to nitrogen gases by heterotrophic denitrifiers in the presence of suitable electron donors (which are commonly organic compounds). Due to the opposing requirements in these processes, nitrification and denitrification are in practice difficult to integrate in one single system and are usually carried out either in separate reactors or in a single reactor system by providing alternate aerobic and anaerobic conditions.

In an attempt to combine effectively these two processes, we have earlier proposed an integrated nitrogen removal system with nitrifying and denitrifying cells co-immobilized in separate layers of double-layer gel beads (16,17, 31,32). The reasoning behind this compact, single system (proposed as the "magic-bead concept") relies on the establishment of aerobic and anoxic zones within the cell supports as a result of oxygen diffusion limitation (18,20). Nitrification occurs in the outer shell whereas in the core denitrification takes place. We have shown that coupled autotrophic nitrification and heterotrophic denitrification could be indeed achieved in air-lift loop reactors at high rates (3-5 kg-N. $m^{-3}_{reactor}.d^{-1}$ at $NH_4^+$ concentrations ranging from 10 to 20 mM) both under steady and dynamic conditions. However, and likewise conventional nitrogen treatment processes, the "magic-bead" system is strongly influenced by the levels of organic carbon because these determine not only the rates of (de)nitrification but also the extent of competing heterotroph growth. Heterotrophic growth influences quite negatively both nitrification and denitrification (17,18,20). Thus, ideally, this system should proceed entirely autotrophically.

Recently, a novel nitrogen removal process (Annamox) was discovered in which ammonium is autotrophically converted to dinitrogen gas under anaerobic conditions with nitrite as electron acceptor (21, 24-27, 33-35). Broda predicted theoretically this anaerobic ammonium oxidation process already in 1977 (4) but it was not until recently that the process has been experimentally discovered (21) and demonstrated to be mediated biologically (33). In that regard, the responsible microorganism has just been identified as a new, very slow growing, autotrophic planctomycete (27). The Anammox process as a whole is given by the following energy-synthesis equation (34,35,26):

$$NH_4^+ + 1.32\ NO_2^- + 0.066\ HCO_3^- + 0.13\ H^+ \Rightarrow$$
$$1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O + 0.066\ CH_2O_{0.5}N_{0.15} \qquad (1)$$

The process was tested in fluidized bed and fixed-bed reactors fed with both anaerobic synthetic medium and sludge digestion effluent (25,34). Conversion rates up 1.5 kg N. $m^{-3}_{reactor}.d^{-1}$ were attained, which shows its great potential for wastewater treatment.
Two potentially important limitations of the Anammox process, however, are the extremely low growth rates of the responsible microorganisms ($\mu_{max} \approx 0.0027\ h^{-1}$) (26,34) and the need of a continuous, roughly equimolar, external supply of nitrite to the system for effective ammonium removal (cf. Eq. 1). Immobilization is likely to provide a way to circumvent the problem posed by the slow-growing microbial cells.

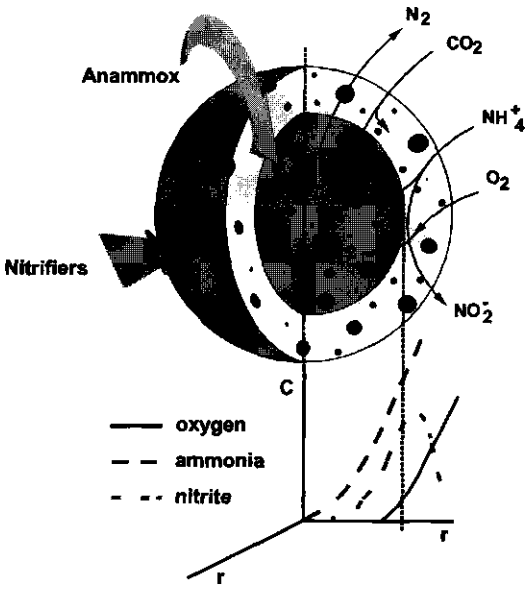


Figure 1 - Representation of the "magic bead" concept applied to the Anammox process (*c* concentration, *r* radius). Outer region: $NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O$; core: $NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$

For the continuous supply of nitrite, an attractive possibility would be the application of the "magic-bead concept" to integrate the Anammox and nitrification processes. Such an application is schematically shown in Figure 1. Ammonium present in the waste streams flows into to the beads where in the outer layers nitrification takes place. The nitrite produced would be subsequently used as electron acceptor for the oxidation of the remaining ammonium in the bead core by the "Anammox cells". Besides its compactness, this system presents the advantage of being completely autotrophic, which results in considerable savings on both organic carbon and oxygen. Additionally, the

absence of organic-carbon in the reactor medium gives much more stability to such a reactor system because it prevents eventual heterotrophic growth of microorganisms present in the wastestreams.

The aim of the research presented here is thus to evaluate and assess the viability of such an integrated system for nitrogen removal. To this end, the Anammox cells were first tested for their performance upon immobilization. Subsequently, removal of ammonium with nitrifying and Anammox cells co-immobilized in double-layer gel beads was assessed both in batch and in continuous reactors.

## MATERIALS AND METHODS

### Microorganisms

*Nitrosomonas europaea* (ATCC 19718) cells were obtained, concentrated and prepared for immobilization as described previously (36). Sludge from a denitrifying fluidized-bed reactor in which anaerobic ammonium oxidation occurred was used as a source of Anammox cells (34). The sludge, which contained about 20 % Anammox cells, was either used immediately or stored at 10°C until needed. Before use, the sludge was homogenized with a blender for 5 minutes.

### Media

The basic medium, used for both the batch and continuous experiments and to store both the cell suspensions and beads contained per litre: $NaHCO_3$, 25 mmol; $KH_2PO_4$, 0.3 mmol, EDTA/$FeSO_4.7H_2O$, 6.3 mg; $CaCl_2.2H2O$, 0.30; $MgSO_4.7H_2O$, 0.20 g; trace-elements solution, 1.25 ml. The trace-elements solution contained (in grams per litre): EDTA, 5.0; $ZnSO_4.7H_2O$, 0.43; $CoCl_2.6H_2O$, 0.24; $MnCl_2.4H_2O$, 1.0; $CuSO_4.5H_2O$, 0.25; $(NH_4)_6MoO_{24}.4H_2O$, 0.22; $NiCl_2.6H_2O$, 0.20; $NaSeO_4.10H_2O$, 0.20; $H_3BO_3$, 0.014; $NaWO_4.2H_2O$, 0.050.

$(NH_4)_2SO_4$ and $KNO_2$ at a concentration of 10 and 5 mmol/l, respectively, were supplemented to all batch experiments, with exception of those containing co-immobilized *Nitrosomonas europaea* and Anammox cells, to which only $(NH_4)_2SO_4$ was added to yield a final concentration of 10 mM during the first 21 days of the run and of 15 mM in the remaining 28 days. KCl and $CaCl_2$ at concentrations of 10 mM were added to the medium used in the continuous experiment to enhance gel strength.

### Immobilization procedure

Co-immobilization of *Ns. europaea* cells and Anammox sludge in separate layers of double-layer beads ("magic-beads" made of carrageenan and alginate gels, respectively) was done by encapsulation as described by Martins dos Santos et al (17,19). A 3.0% (w/v) alginate gel solution containing Anammox sludge and 0.75 M KCl was extruded

drop wise through a hollow needle (2 mm in diameter) into a 1.5 % (w/v) stirred κ-carrageenan solution containing the *Ns. europaea* cells and 0.2 M $CaCl_2$. Beads with an external diameter of 6.1 ± 0.4 mm and with a core diameter of 4.4 ± 0.2 were obtained (Figure 2).

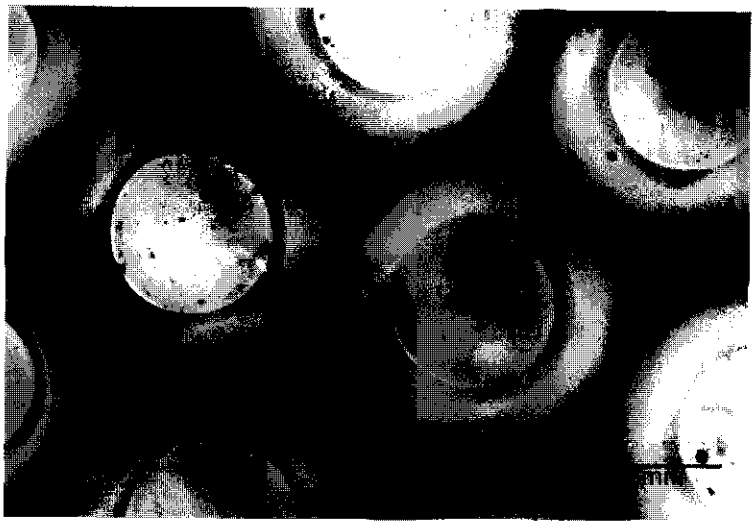

Figure 2 - Double-layer gel beads ($\varnothing_{core}$ = 4.4 ±. 0.3 mm, $\varnothing_{total}$ = 6.1 ±. 0.2 mm).

**Suspended and immobilized cultivation of cells**

***Batch experiments***

Removal of nitrogen compounds by *Ns. europaea* and Anammox sludge was assessed by cultivating these microorganisms, both as single as a mixed cultures, in batch at 30°C, with gentle agitation (100 rpm) in a rotary shaker. The cells were cultivated both in suspension and immobilized in gel beads, and both under anoxic and aerobic conditions (Table 1). All experiments were done in quadruplicate. Control experiments with bottles containing all components except the cells were tested for each combination.

Table 1 – Sets of batch experiments done with *Ns. europaea* and Anammox cells

| | **Anaerobic** | **Aerobic** |
|---|---|---|
| **Suspended** | *Ns. europaea* | *Ns. europaea* |
| | Anammox | Anammox |
| **Immobilized** | *Ns. europaea* | *Ns. europaea* |
| | Anammox | Anammox |
| **Co-Immobilized** | *Ns. europaea* +Anammox | *Ns. europaea* +Anammox |

*Single populations*

Anaerobic batch experiments with cell suspensions and single beads were carried out in 120 ml serum-bottles containing basic medium supplemented with 10 mM $(NH_4)_2SO_4$ and 5 mM $KNO_2$. Concentrated cell suspensions (3.0 ml *Ns. europaea* or 20.0 ml Anammox) were added to the medium so that the final liquid-phase volume was 100.0 ml. Both the total liquid-phase and headspace volume (about 15.8 ml) was determined accurately for every bottle. The bottles were tightly closed with 12-mm thick butyl rubber septa. To obtain an air-free atmosphere, the medium in each bottle was flushed with helium for 15 minutes and then placed in an automatic gas-exchange device (Anaeroob Lap-Systeem, Wageningen Agricultural University, Dept. Microbiology). In this device, vacuum was created in the bottles, which were subsequently flushed with helium from 0.8 bar underpressure to 0.2 bar overpressure, with a final overpressure of 0.2 bar in each bottle. This was done for each bottle 24 times.

One ml of 1 M $KHCO_3$ was injected to each flask giving an initial concentration of approximately 10 mM, to provide additional buffering as most of the $CO_2$ in the buffer medium would have been removed during flushing. Aerobic batch experiments were prepared as the anaerobic ones except that the 100.0 ml liquid phase was placed in 250-ml serum bottles closed with cotton plugs.

*Co-immobilized cells*

Batch experiments with co-immobilized cells were carried out both under aerobic and anoxic conditions in a similar way as described for singly immobilized cells. Hundred ml of buffer medium and 50 ml of beads were placed into eight 250-ml bottles. Two control bottles containing only medium were prepared as well. All bottles were tightly sealed with 12-mm thick butyl rubber septa and made anaerobic as described above. 1.5 ml of 0.5 M $(NH_4)_2SO_4$ was injected into each bottle, giving an initial $NH_4^+$ concentration of approximately 10 mM. One and a half ml of 1 M $KHCO_3$ was injected to each flask giving an initial concentration of approximately 10 mM, to provide additional buffering. At time zero 7.5 ml of oxygen (5% of the original head-space volume) was injected into four of the inoculated bottles and into one of the control flasks. Another 15 ml of oxygen (10% of the head-space volume) was injected after 1 week and a further 30 ml of oxygen (20% of the head-space volume) was injected after two weeks. 0.75 ml of 1 M $NaNO_2$ was injected into four of the bottles - the anaerobic bottles - and 0.5 ml into the other control flask to give an initial $NO_2^-$ concentration of approximately 5 mM. Similar batch experiments were done at the end of the continuous runs with the co-immobilized cells. Ammonium and oxygen were injected repeatedly during the batch experiments as soon as no oxygen or ammonium was left in the bottles.

For all batch experiments, liquid samples (1ml) were taken each day using a Benton-Dickinson Plastipac 1 ml syringe and centrifuged in a Micro Cen13 microfuge (Herolab GmbH Laborgeräte) for 5 minutes and then placed in the refrigerator at 4°C until analyzed for nitrite and ammonium. In the batch experiments involving co-immobilized cells, nitrate was analyzed as well. The pH of the liquid samples was also measured and additional 1 M $KHCO_3$ added as required to maintain a pH suitable for cell growth (7.3 - 8.3).

In all batch experiments carried out under anaerobic conditions and in those with co-immobilized cells (thus both under aerobic and anaerobic conditions), duplicate 300 µml gas samples for gas analysis were taken each day using a 500 µml pressure-lock SGE syringe with a 23G needle and an SGE series II repeating adapter.

***Continuous experiments***

A continuous internal air-lift loop reactor was set up in a similar way as earlier described (18). The pH was maintained within the range 7.46-7.70 by a E+H Conducta Liquisys-P pH controller using 0.25 M HCl and 0.25 M KOH, and the temperature maintained at 30°C by means of water jackets around the reactors. The working volume of the reactor was 483 ml of which approximately 20% consisted of beads. The beads were the same as those used in the batch experiment. Medium was pumped into the reactor with a retention time of approximately 3.3 hours by a Watson and Marlow 101U pump. Liquid samples were taken (using disposable 1-ml syringes) each day from the effluent and influent and were analyzed for nitrite, nitrate and ammonium. At the end of the experiments, part of the beads were transferred to serum flasks and cultivated aerobically in batch with medium containing ammonium but no nitrite. The composition of both the liquid and gas phase was monitored by sampling periodically. At the end of the batch cultivation, the flasks were supplemented with fresh medium, made oxygen and nitrogen free and cultivated again for about three days. After that time, 5 mM of nitrite were injected into the flask, which was subsequently incubated for another 3 days. This whole set of experiments was done twice.

**Analytical methods**

***Analysis of solutes and biomass***

Ammonium, nitrite and nitrate concentrations in batch media and in the influent and effluent of the reactors were measured by colorimetrically as described elsewhere (9). Biomass concentrations were estimated from dry-weight measurements and biological oxygen monitoring. For the dry-weight measurements, six aluminium foil dishes were placed at 100 °C for 24 hours after which they were weighed. 5 ml of resuspended *Nitrosomonas* cells were added to each of three of the dishes and 5 ml of blended

Anammox sludge was added to each of the three remaining dishes. The six dishes were then individually weighed, placed back in the oven at 100°C for 48 hours and then reweighed. The weight of biomass was corrected for the weight of the salts present in the sample, which was determined by ashing. The activity of *Nitrosomonas europaea* cells was measured when (i) the cells were in suspension and (ii) after immobilization into double beads, using a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Incorporated, Ohio 45387 USA) with the chamber surrounded by thermo-stated water at a temperature of 30°C. The measurements were done as earlier described (18).

***Gas analysis***

Nitrogen, oxygen and nitrous oxide concentrations were measured using a gas chromatograph as described before (17). The results were corrected both for dilution due to sampling and for the change in pressure due to gas production and consumption.

## RESULTS

### Conversion of N-compounds by suspended cells

Prior to their immobilization, *Ns. europaea* and Anammox sludge were separately tested, in suspension, for their ability to remove nitrogen compounds during batch cultivation, both under aerobic and anaerobic conditions. Under aerobic conditions (Figure 3a), the nitrifying cells converted ammonium and produced nitrite at an average rate of about 0.06 $mmol.l^{-1}.hr^{-1}$ over the first 100 hours.

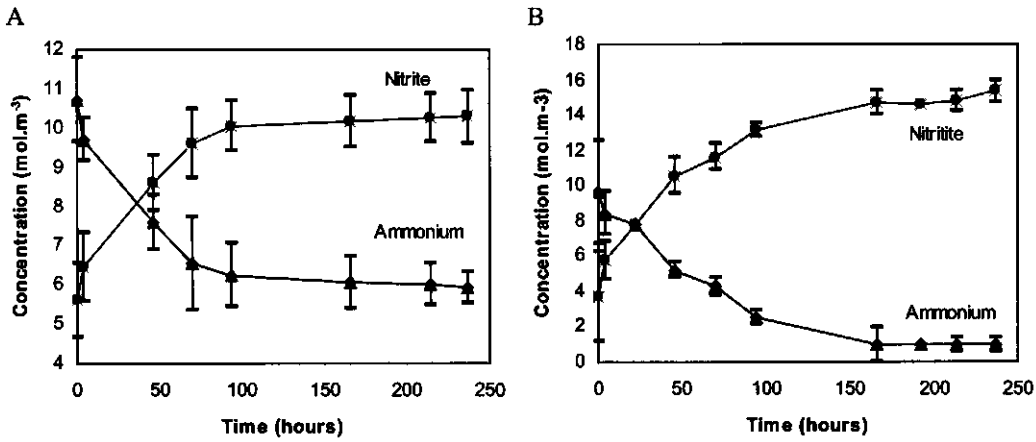


Figure 3 - Aerobic batch cultivation of *Nitrosomonas europaea* cells: a) in suspension; b) immobilized in κ-carrageenan beads.

The total ammonium removed in each flask matched the amount of nitrite produced throughout the whole experiment, which excludes a possible reduction of nitrite to

nitrate or to nitrogenous gases. The pH fell from 7.6 to about 6.4 in the first 150 hours, which, very likely, prevented the cells from further converting the remaining ammonium. Practically no ammonium was consumed nor any nitrite or nitrogen gas produced under anoxic conditions (results not shown). However, small amounts of nitrous oxide were detected (up to about 0.02 $mmol.l^{-1}_{gas}$, $V_{gas}/V_{liq} \sim 0.16$ ).

Under anoxic conditions, the Anammox sludge consumed both ammonium and nitrite at a ratio of 1 to 1.5 (Figure 4a), which is slightly higher than that predicted by equation 1 (1:1.32). The sludge produced nitrogen up to a final concentration of 12.3 $mmol.l^{-1}_{gas}$ in the gas phase. At the end of the experiment nitrous oxide was produced up to a concentration of about 0.15 $mmol.l^{-1}_{gas}$ (not shown). Most of the nitrogen production activity took place between 100 and 160 hours after start-up. The pH increased slightly from 7.8 to 7.9. A nitrogen balance was made at each time step with $NH_4^+$, $NO_2^-$, $N_2$ and $N_2O$ concentrations, taking into account the dilution of both the gas and liquid phases due to sampling and considering the change in pressure due to production and consumption of gas. After doing so, the amount of nitrite and ammonium consumed matched the amount of gas (both $N_2$ and $N_2O$) produced within about 3-4 % of the total nitrogen, without taking into account the nitrogen fixed into biomass.

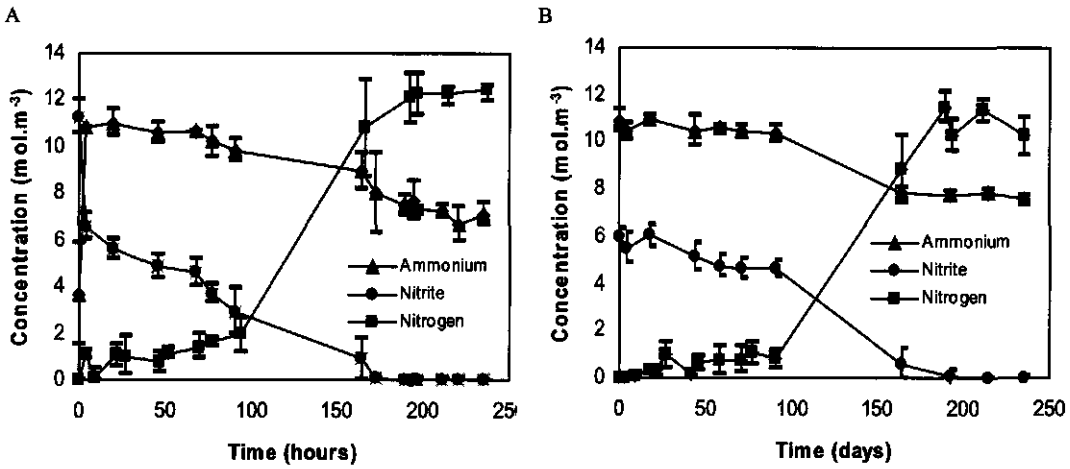


Figure 4 - Anaerobic batch cultivation of Anammox sludge: a) in suspension; b) immobilized in alginate beads.

In the aerobic bottles containing Anammox sludge, ammonia was partly converted into nitrite, although at a rate twice as low as that of the nitrifying bottles (results not shown). The pH fell from 8.2 at the beginning of the experiment to 7.1 at the end. Jetten and co-workers (11, 24, 34) reported the presence of nitrifying cells in Anammox sludge, which likely explains the nitrification observed under these aerobic conditions.

**Conversion of N-compounds by singly immobilized cells**

Immobilized *Ns. europaea* cultivated under aerobic conditions converted ammonium into nitrite at a rate of 0.088 $mmol.l^{-1}.h^{-1}$ (Figure 3b). The nitrogen balance remained constant at about 15 $mmol.l^{-1}$. The pH fell from 8.2 to 6.6 after the first 150 hours which, likely, prevented the cells from further converting the remaining ammonium (about 1 mM).

Ammonium and nitrite were consumed simultaneously in the flasks containing Anammox cells immobilized in alginate beads cultivated under anoxic conditions (Figure 4b). Most of the conversion took place between 90 and 160 hours after the start-up and nitrite decreased at a rate approximately 1.5 times faster that that of ammonium. After all nitrite disappeared, no ammonium was consumed. The pH increased slightly from 8.0 to 8.1. During the experiment, dinitrogen accumulated in the gas phase up to a final concentration of about 20 $mmol.l^{-1}$. A little nitrous oxide was produced in all four flasks and in two of them a peak concentration of 1.3 $mmol.l^{-1}_{gas}$ was observed, after which its concentration decreased to 0.6 $mmol.l^{-1}_{gas}$. In the other two flasks the $N_2O$ concentration increased up to about 0.3 $mmol.l^{-1}_{gas}$. The nitrogen balance at each time step was closed within 3-4 % of the total nitrogen present in the bottles.

**Conversion of N-compounds by co-immobilized cells**

Figure 5 shows the results of aerobic cultivation of *Ns. europaea* and Anammox immobilized in the outer and inner layers of "magic-beads", respectively. The concentration of ammonium decreased from approximately 10 mM at the beginning of the experiment to 1 mM at the end, whereas $NO_2^-$ was formed. This indicated that the *Ns. europaea* was active and converting the ammonium and oxygen into nitrite. The arrows indicate oxygen injections. These two extra injections were made after observing that the oxygen concentration did not decrease further, suggesting that the nitrifying cells would have been severely limited by oxygen (Figure 5b). Indeed, an immediate decrease in the oxygen concentrations followed the two injections. It should be stressed that the decrease in oxygen tensions was even sharper than those observed in Figure 5b because the flasks were just sampled the day after the injections. These injections would have increased the oxygen level to 5.4 and 8.9 $mmol.l^{-1}_{gas}$, respectively. The reductions in oxygen concentration following the injections were accompanied by a corresponding decrease in ammonium and a sudden increase in nitrite. The subsequent reduction in the nitrite concentration indicated that the Anammox sludge was active and that it converted nitrite and ammonium into nitrogen gas. Regularly, small amounts of nitrate were found. $N_2O$ was occasionally detected at negligible levels. The nitrogen balances closed within 4-5% of the total nitrogen.

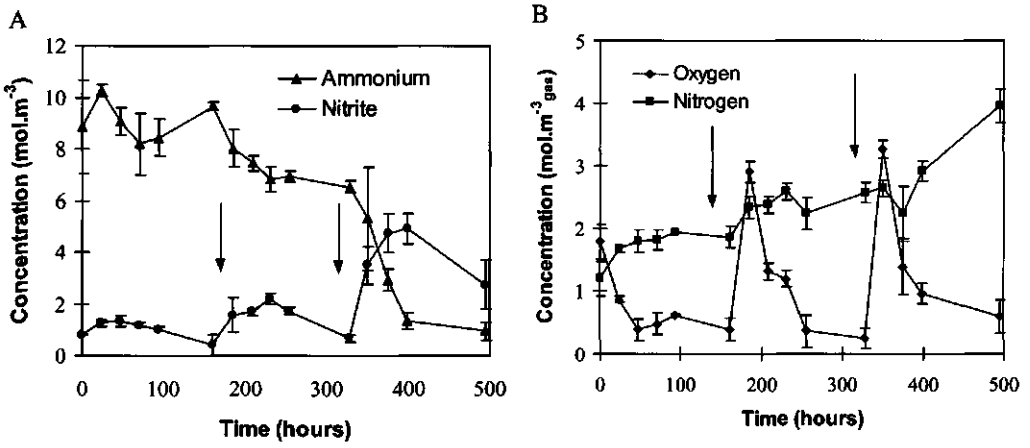


Figure 5 - Aerobic batch cultivation of *Nitrosomonas europaea* cells and Anammox sludge co-immobilized in separate layers of "magic beads". Concentration of N-compounds in: a) liquid phase; b) gas phase. Arrows indicate injections of oxygen.

In parallel with these aerobic experiments, anoxic flasks (to which nitrite had been added) containing magic-beads were run to verify whether the Anammox process was indeed occurring within the core of the double-layer beads. In these flasks, both ammonium and nitrite decreased with time at rates similar to those observed in the flasks with singly immobilized Anammox sludge, whereas nitrogen gas accumulated (results not shown). Occasionally, traces of nitrous oxide were detected as well. Towards the end of the experiments, both in the aerobic and anaerobic flask, gas bubbles (presumably of nitrogen) were visible in between the inner and outer layers of some beads. These bubbles remained within the beads even after storing them for more than two months.

**Continuous ammonium removal with magic-beads**

At the end of the aerobic batch experiments, part of the magic-beads was collected and transferred to a continuously operated air-lift loop reactor. The run is shown in Figure 6. A nitrogen balance was made relating the moles of ammonium and the moles of nitrite and nitrate produced. The activity of Anammox sludge was estimated on the basis of the nitrogen "missing" between the ammonium consumed and the nitrite, correcting for the nitrate that would have been formed due to Anammox activity (cf. Eq.1). Activity of *Ns europaea* was calculated by "adding" the nitrite that would have been consumed by Anammox (cf. Eq1) to the nitrite effectively found in the bulk. In these calculations, it was assumed that the 'missing nitrogen' was due to nitrogen gas production.

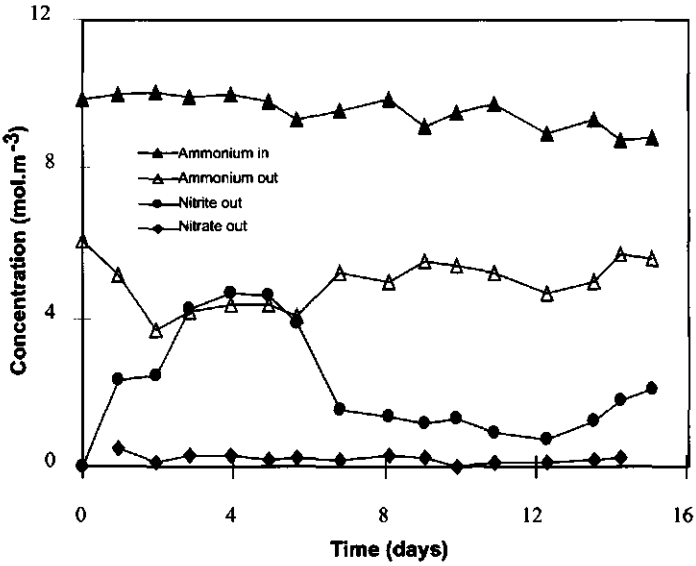


Figure 6 - Continuous cultivation of the "magic beads" (taken out of the batch experiment, Figure 5) in an air-lift loop reactor.

This assumption was used since the results of the batch experiment had shown that nitrogen gas was produced as a result of anaerobic ammonium removal. In addition, large bubbles were visible in an increasingly large number of beads during the first few days of the experiment. After these first 4-5 days many beads began to loose their outer layers, likely due to pressure build-up caused by the gas bubbles. Towards the end of the experiment, only approximately on third of the initial bead had not burst. This implied that, although the reactor overall nitrogen removal rates progressively decreased from a maximum of 0.6 $kgN.m^{-3}_{reactor}.day^{-1}$ to 0.2 $kgN.m^{-3}_{reactor}.day^{-1}$, the specific removal rates per gel volume remained approximately constant at 3 $kgN.m^{-3}_{gel}.day^{-1}$ (Figure 6). After 15 days the continuous experiment was stopped and the remaining double-layer gel beads were transferred to serum flasks and tested in batch for their ability to remove ammonia. The results are shown in Figure 7. Both ammonia (7a) and oxygen (7b) were consumed very rapidly in the first 10 hours. Both the ammonium consumption and nitrogen production rates attained were higher than those measured before the continuous run (Figure 5). A little nitrite was produced as well as about 2 $mmol.l^{-1}$ nitrogen, but no nitrous oxide was detected. Traces of nitrate were found as well. The system responded rapidly in the same way after injection of oxygen and ammonium at about 23 hours after start up. Repetition of this whole set of experiments yielded comparable results.

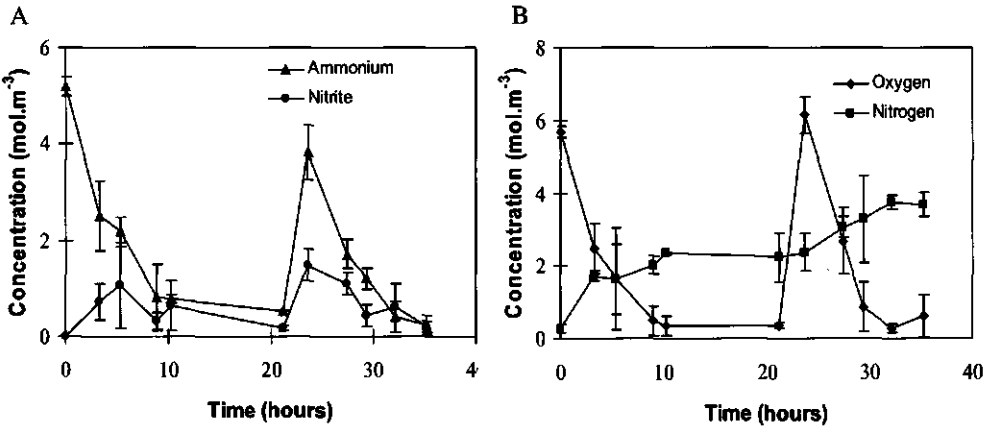


Figure 7 - Aerobic batch cultivation of the "magic beads" taken out the continuous reactor (Figure 6). Concentration of N-compounds in: a) liquid phase; b) gas phase.

## DISCUSSION

### *Nitrogen removal*

The aim of this work was to develop a compact, fully autotrophic system for nitrogen removal using "classical" nitrifiers and anaerobic ammonium-oxidizing cells co-immobilized in double-layer beads. For this, we assessed first the capacity of "anammox" microorganisms to remove ammonium and nitrite both in suspension and in beads. In these experiments, both suspended and singly immobilized Anammox-sludge did indeed convert ammonium and nitrite anaerobically into nitrogen gas at similar rates (Figure 4). Clearly, entrapment within a gel matrix did not affect the Anammox process. Van de Graaf and co-workers (34) showed that, due to its low growth rates, continuous growth of the sludge responsible for Anammox required some form of biomass retention, in particular if used for the treatment of high flows of diluted streams (10, 11, 25, 26). That the Anammox sludge was not visibly affected by immobilization gave good perspectives for its co-immobilization with *Ns. europaea*, which would then provide the nitrite necessary for ammonium oxidation. These perspectives were supported by the findings of Strous et al. (24) who showed that the Anammox process was only reversibly inhibited by oxygen. This means that temporary exposure of Anammox sludge to oxygen (as for instance, during start-up) would not permanently affect nitrogen removal in a system with co-immobilized cells.

Furthermore, the batch experiments with suspended *Ns. europaea* indicated that, at least within the time span of the experiments, these cells were only able to remove ammonium at significant rates (basically, by converting it into nitrite) under aerobic conditions. Studies reporting on the anaerobic metabolism of *Nitrosomonas* spp. (1, 3, 10, 13, 23) have shown that, although in some circumstances (for instance, under

oxygen stresses) these cells could use nitrite as electron acceptor for ammonium oxidation, their rates of combined nitrification and denitrification were at least two or three orders of magnitude lower than those of normal nitrification under aerobic conditions. In addition, Strous and co-workers have recently (24) demonstrated that the nitrifying microorganisms present in the Anammox sludge (*Nitrosomonas* spp. and *Nitrobacter* spp.) do not play an important role in the conversion of ammonium under anaerobic conditions.

This set of results as a whole implies that the nitrogen produced in the experiments involving co-immobilized Anammox and *Ns. europaea* likely resulted from the Anammox activity. The accumulation of gas bubbles between the inner and outer layers of double-layer beads supports such conclusion. Since no nitrite or nitrate were added to the media, these results clearly indicate that coupled autotrophic nitrification by nitrifying bacteria and anaerobic ammonia oxidation by the Anammox sludge took place in the beads. The rates achieved in the reactor (about 3 $kgN.m^{-3}_{gel}.day^{-1}$ in both continuous runs) were comparable to those of nitrification or denitrification alone in reactor systems based either on artificially entrapped microorganisms (12, 22, 30, 36-38) or naturally attached biofilms (28, 29). The rates were also similar to those obtained earlier (21, 25, 34) for the anaerobic oxidation of ammonium by Anammox sludge in fluidized bed reactors supplied with both ammonium and nitrite.

***Dynamics***

That a fundamentally aerobic process (nitrification) and a strictly anaerobic one (Anammox) can take place simultaneously within a bead can be attributed to the establishment of aerobic and anaerobic regions across the particles as a result of oxygen diffusion limitation. We have shown that this is indeed so by measuring the oxygen, nitrite and ammonium concentration profiles across a gel matrix with co-immobilized autotrophic nitrifiers and heterotrophic denitrifying cells (20). We have also shown that the extent and rate at which oxygen is depleted from the inner bead layers depends strongly on the activity of the aerobic cells (9, 18, 20 and 37). Hence, the accumulation of nitrite (up to 5 mM) in the reactor effluent (Figure 6) during the first part of the experiment indicated clearly that the aerobic nitrifying activity was not high enough to prevent oxygen to reach the deeper beads layers where Anammox was present. From day four onwards nitrite decreased progressively suggesting that diffusion limitation became effective in protecting Anammox from oxygen. Such an oxygen-protecting effect has a general character (as it results from the activity of aerobic cells in the outer layers) and has been frequently reported for different systems (2, 5-7, 14 and 16-18). Computer simulations (not shown here) using a (validated) dynamic model that describes growth of immobilized (de)nitrifying cells (16, 20) show that, for the

experimental conditions of this study, oxygen would indeed fully penetrate the bead until about day four. From then on anoxia in the core prevails, which enables the Anammox cells to become active. These results show clearly that the whole process is regulated by activity of the nitrifying cells immobilized on the outer layers. The anaerobic ammonium oxidation depends on them in three ways: a) the oxygen not used by the nitrifiers; b) the nitrite produced and c); the ammonia left unoxidized. Effective nitrogen removal by the system proposed here would thus require a careful design and control of the operating parameters, in particular of those related to the oxygen and ammonium concentration in the reactor.

***Perspectives***

The system proposed in this study (Magic Bead - Anammox) presents, besides its compactness the advantage of being fully autotrophic. Such a system allows considerable resource savings as compared to conventional processes because: a), no organic carbon is needed and b) about 50% of the oxygen can be saved (25% by avoiding intermediate nitrate formation and 25% because only half of the total ammonium needs to be oxidized aerobically). These savings in oxygen are especially important because the energy costs due to aeration can be strongly reduced. In addition to these savings, the absence of organic-carbon in the influent medium gives much more stability to such a reactor system because it prevents heterotrophic growth of microorganisms present in the wastestreams. Since just half of the ammonium is oxidized aerobically, the acid generated during this process will be also the half of that normally produced. The pH control will be thus greatly facilitated, especially if the streams to be treated are basic themselves (e.g., effluents from anaerobic digesters).Finally, both being very slow autotrophic growers and having a low biomass yield (28), sludge production is negligible, reducing enormously the disposal of biomass surplus. In conventional nitrogen removal processes a large fraction of the excess sludge is due to the activity of the heterotrophic denitrifiers (10, 11, 26, 28).

In a sense, this system can be considered almost perfect (in particular for highly loaded wastewaters) because they fulfill most of the requirements commonly accepted as essential in future wastewater-treatment processes (8, 10, 15, 19). Yet, before this process is to be used in effective nitrogen removal, some hurdles should be overcome. To our view, one of the most important is the stability of the immobilization support itself since both continuous runs had to be stopped at a relatively early stage due to the desegregation of the bead envelopes. We have addressed this issue in a separate paper, where several combinations of immobilization techniques and support materials were presented for the production of stable double-layer beads (19). In that regard, Leenen and co-workers (15) have shown quantitatively that the mechanical stability of

biocatalyst supports is crucial for the application of immobilized-cell technologies at full scale in wastewater-treatment processes.

In conclusion, we believe that, providing that an appropriate combination of support material-immobilization technique is chosen and that the system is well designed, the process proposed here can contribute significantly for the effective removal of nitrogen from wastewaters, either as a post-treatment step or directly from streams with low organic-carbon content.

## ACKNOWLEDGEMENTS

Financial support by the Junta Nacional de Investigação Científica e Tecnológica (JNICT), Portugal is gratefully acknowledged.

## REFERENCES

1. Abeliovich A. and A. Vonshak. 1992. Anerobic metabolism of *Nitrosomonas europaea.* Arch. Microbiol. 158:267-270.
3. Beunik H, Rehm HJ. 1990 Coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol by a co-immobilized mixed culture system. Appl. Micro. Biotech. 34:108-115
4. Bock, E., I. Schmidt, R. Stuven and D. Zwart. 1995. Nitrogen loss caused by denitrifying *Nitrosomonas* cells using ammonium or hydrogen as electron donors and nitrite as electron acceptor. Arch. Microbiol. 163:16-20.
4. Broda, E. 1977. Two kinds of microorganisms missing in nature. Z. allg. Mikrobiol. 17:491-493.
5. Field J.A., Stams A.J., Kato M., Schraa G. 1995. Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. Antonie van Leeuwenhoek. 67:47-77
6. Gardin H., Pauss A. 1994. Co-immobilization of aerobic and anaerobic bacterial communities in a mixed κ-carrageenan/gelatin gel for the degradation of 2,4,6-thrichlorophenol, Proc. International Workshop Bioencapsulation IV, Quebec, Canada , pp. 93-97
7. Gerritse J. and Gottschal J.C. 1992. Mineralization of the herbicide 2,3,6-trichlorobenzoic acid by a coculture of aerobic and anaerobic bacteria. FEMS Microbioly Ecology, 10:89--98
8. Heijnen, J.J, van Loosdrecht, M.C.M., Mulder R., Weltevrede, R., Mulder, A. 1991. Large scale anaerobic-aerobic treatment of complex industrial wastewater using biofilm reactors. Wat. Sci. Technol., 23:1427-1436
9. Hunik J.H., Bos C.G., Van Den Hoogen P., De Gooijer C.D., Tramper J. 1994a. Co-immobilized *Nitrosomonas euroapaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in k-carrageenan gel beads. Biotechnol. Bioeng., 43:1153-1163.

10. Jetten, M.S.M., S. Logemann, G. Muyzer, S. de Vries, M.C.M. van Loosdrecht, L.A. Robertson and J.G. Kuenen. 1997a. Novel principles and processes in the removal of nitrogen from wastewater. Antonie van Leeuwenhoek 71:73-95

11. Jetten, M.J.M., Strous, M., van de Graaf, A.A., Logemann, S., Muyzer, G., van de Pas-Schoonen, Schalk, J., van Loosdrecht, M.C.M. and J.G. Kuenen,. 1999. The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. 22:421-437

12. Kokufuta E., M. Shimoshashi and I Nakamura. 1987. Continuous column denitrification using polyelectrolyte complex-entrapped *Paracoccus denitrificans* cells. J. Ferment.Technol. 65:359-361

13. Kuenen, J.G. andL.A. Robertson. Combined nitrification-denitrification processes. FEMS Microbiol. Revews. 15:109-117

14. Kurosawa H. and H. Tanaka. 1990. Advances in immobilized cell culture: development of a co-immobilized mixed culture system with aerobic and anaerobic microorganisms. Process Bioche. Int. 25: 189-196.

15. Leenen, E.J.T.M., Martins dos Santos, V.A.P., Grolle, K.C.F., Tramper, J., Wijffels, R.H. 1996. Characteristics of and selection criteria for cell immobilization in wastewater treatment.Water Res. 30: 2895-2996.

16. Martins dos Santos V.A.P., J. Tramper and R.H. Wijffels. 1993. Simultaneous nitrification and denitrification using immobilized micro-organisms. Biomat. Artif. Cells & Immob. Biotechnol. 21: 317--322

17. Martins dos Santos V.A.P., M. Bruijnse, Tramper J., Wijffels R.H. 1996a. The Magic-Bead concept: an integrated approach for nitrogen removal. Appl. Microbiol. Biotechnol. 45:447-453.

18. Martins dos Santos V.A.P., J.L. Marchal, J. Tramper J, R.H. Wijffels. 1996b. Modeling and evaluation of an integrated nitrogen removal with co-immobilized micro-organisms in double-layer gel beads. Biotechnol. Progress. 12: 240-248.

19. Martins dos Santos V.A.P., Vasilevska T., Kajuk, B., Tramper J., Wijffels R.H. 1997. Production and Characterization of Double-Layer Beads for Co-Immobilization of Microbial Cells. Biotechnology Annual Review, 3:227-244

20. Martins dos Santos V.A.P., Tramper J., Wijffels R.H. 1999. Substrate and product profiles across double-layer gel geads: modelling and experimental evaluation. Submitted for publication.

21. Mulder, A., A.A. van de Graaf, L.A. Robertson and J.G. Kuenen. 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. 16:177-184.

22. Nilsson I. and S. Ohlson. 1982. Columnar denitrification of water by immobilized cells. Appl. Microbiol Biotechnol. 14: 86-90.

23. Poth, M. 1986. Dinitrogen production from nitrite by a *Nitrosomonas* isolate.Appl. Environ. Microbiol. 52:957-959.

24. Strous, M., E. van Gerven, J. G. Kuenen and M.S.M. Jetten. 1997a. Effect of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (Anammox) sludge. Appl. Environ. Microbiol. 63:2446-2448.

25. Strous, M., E. van Gerven, P. Zheng, J. G. Kuenen and M.S.M. Jetten. 1997b. Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (Anammox) process in different reactor configurations. Wat. Res. 31:1955-1962.

26. Strous, M. M., E. van Gerven, J. G. Kuenen and M.S.M. Jetten. 1998. The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl. Microbiol. Biotechnol. 50:589-596

27. Strous, M.,Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K., Webb, R., Kuenen, J.G. and M.J.M Jetten. 1999. Missing lithotroph identified as new plactomycete. Nature 400,446-449

28. Tijhuis L. J.L. Huisman, H.D. Hekkelman, M.C.M. van Loosdrecht and J.J. Heijnen. 1994. Formation of nitrifying biofilms on small suspended particles in airlift reactors. Biotechnol. Bioeng. 47:585-595.

29. Tanaka H. and I. J. Dunn. 1981. Kinetics of nitrification using a fluidized sand bed reactor with attached growth. Biotechnol. Bioeng. 23:1683-1702.

30. Tanaka K., T. Kimata, S. Harada, Y. Fujii, T. Mizuguchi, N. Mori and H. Emori. 1991. Development of new nitrogen removal systems using nitrifying bacteria immobilized in synthetic resin pellets. Water Sci. Technol. 23:681-690.

31. Tramper, J. Nitrification and denitrification by immobilized bacteria. 1984. Proc. 3rd European Congress on Biotechnology. VCH, vol. IV:363-368.

32. Tramper J. 1987. Nitrification and denitrification by immobilized viable cells. *In*: A.I. Laskin, L. Mosbach, D. Thomas, L.B. Wingard (eds.) Enzyme Engineering *8*, Annuals of the New York Academy of Sciences. 501: 362-366

33. van de Graaf, A.A. A. Mulder, P. de Bruijn, M.S.M. Jetten, L.A. Robertson and J.G. Kuenen. 1995. Anaerobic ammonium oxidation is a biologically mediated process. Appl. Environ. Microbiol. 61:1246-1251.

34. van de Graaf, A.A. A. Mulder, P. de Bruijn, M.S.M. Jetten, L.A. Robertson and J.G. Kuenen. 1996. Autotrophic growth of anaerobic ammonium oxidizing microorganisms in a fluidized bed reactor. Microbiology (UK). 142:2187-2196.

35. van de Graaf, A.A. A. Mulder, P. de Bruijn, L.A. Robertson, M.S.M. Jetten and J.G. Kuenen. 1997. Metabolic pathway of anaerobic ammonium oxidation on the basis of $^{15}N$ studies in a fluidized bed reactor. Microbiology (UK). 143:2415-2421.

36. Wijffels R.H. and J. Tramper. 1989. Performance of growing *Nitrosomonas europaea* cells immobilized in carrageenan. Appl. Microbiol. Biotechnol. 32: 108-112.

37. Wijffels R.H., De Gooijer C.D., Kortekaas S., Tramper J. 1991. Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in κ-carrageenan. Part 2: Model evaluation. Biotechnol. Bioeng. 38: 232-240

38. Wijffels R.H., Schukking A, and J. Tramper. 1990. Characterization of a denitrifying bacterium immobilized in κ-carrageenan, Applied Microbiol. Biotechnol. 34:399-403

This chapter has been submitted for publication by Martins dos Santos VAP, Holland A, Jetten, Kuenen JG, Tramper J, Wijffels RH.

## CHAPTER 11

# Process Dynamics of a Fully Autotrophic Nitrogen Removal System


## ABSTRACT

A compact, fully autotrophic single-staged system is proposed to combine "classical" aerobic nitrification by autotrophic nitrifying cells with the recently discovered anaerobic ammonium-oxidation process (Anammox). The system takes advantage of the opposite oxygen requirements of the nitrifying (strict aerobes) and Anammox (anaerobes) cell populations. In this combined process, ammonium is partly oxidized by nitrifying cells co-immobilized in gel beads as a mixed culture with Anammox sludge. Due to the fast oxygen uptake by the nitrifiers, both biomass and substrate profiles develop leading to the establishment of extensive anoxic zones within the beads. The nitrite produced by the aerobic nitrifiers and the remaining ammonium are then anaerobically converted into nitrogen by the Anammox microorganisms in the anoxic zones. This system was shown to work well both in batch and in continuous mode. Overall nitrogen removal rates up to 1.5 kg-N.$m^{-3}$.$day^{-1}$ were achieved in continuously driven air-lift loop reactors.

To gain insight into the mechanisms underlying the processes involved and to be able to design further applications of the system, a dynamic model describing the interactions among nitrifying (*Nitrosomonas* spp. *and Nitrobacter* spp.) and Anammox microorganisms co-immobilized within single-layered gel beads was developed. The model predicted adequately the experimentally measured behavior of the system (growth, conversion of substrates and mass transport) both under steady and dynamic conditions. The proposed system is highly compact and reduces greatly the energy and organic-C inputs. Therefore, it may be a valuable alternative for effective nitrogen removal from wastewaters.

## INTRODUCTION

Recently, a compact, fully autotrophic single-staged system was proposed in which "classical" aerobic nitrification by autotrophic nitrifying cells was combined with the newly discovered ANAerobic aMMonium OXidation process (ANAMMOX, van de Graaf *et al.*, 1995,1996, Strous *et al.*, 1998, 1999). The system is based on the "magic-bead" concept, in which nitrifying and anaerobic ammonium-oxidizing microorganisms are co-immobilized in double-layer gel beads (Martins dos Santos, 1996a, 2001). In this process, ammonium is partly oxidized by nitrifying cells immobilized in the outer layer of the beads. The nitrite produced by the aerobic nitrifiers and the remaining ammonium are then anaerobically converted into nitrogen by the Anammox microorganisms entrapped in the bead core. This system was shown to work well both in batch and in continuous mode and it seemed quite promising as an alternative for effective removal of nitrogen from wastewater.

A major drawback of the system, however, is that the production of the double-layered biocatalyst particles is intrinsically more troublesome than that of single beads. In fact, the magic-bead concept was originally developed to force habitat segregation between "classical" nitrifiers (strict aerobes) and heterotrophic denitrifiers (facultative anaerobes). Such forced segregation prevents the former to be outgrown by the latter due to competition for oxygen (Martins dos Santos *et al.*, 1996a). Yet, van de Graaf and co-workers (1995,1996, Strous *et al.*, 1997a, 1999) have clearly shown that the ammonium oxidation by Anammox cells is strictly anaerobic. Thus, at least in principle, it should be possible to obtain fully autotrophic ammonium oxidation by co-immobilizing a mixed culture of nitrifying and Anammox sludge in single gel beads.

Hence, in this paper we report on the development and design of such a compact, fully autotrophic system for removal of nitrogen from wastewaters. To gain insight into the mechanisms underlying the processes involved and to be able to design its applications, a dynamic model, previously developed and validated for simultaneous autotrophic nitrification and heterotrophic denitrification in magic beads (Martins dos Santos *et al*, 1996b, 2001), was adapted to include the sludge responsible for the Anammox process. Accordingly, this model was extended to describe the simultaneous growth of the three competing microbial populations (*Nitrosomonas* spp., *Nitrobacter* spp., and Anammox) co-immobilized as a mixed culture within single gel beads and run in a continuous airlift bioreactor.

## MATERIALS AND METHODS

### Microorganisms

For batch experiments with co-immobilized cells, a pure culture of *Nitrosomonas europaea* was used. For the continuous experiments and to study the interactions between the cell populations, a nitrifying sludge containing about 98% *Nitrosomonas* spp. and 2 % *Nitrobacter* spp. cells obtained from a nitrifying reactor was used. Sludge from a denitrifying fluidized-bed reactor in which anaerobic ammonium oxidation occurred was used as a source of Anammox (van de Graaf *et al.* 1995). The sludge, which contained about 20 % Anammox cells, was either used immediately or stored at 10°C until needed. Before use, the sludge was homogenized with a blender for 5 minutes.

### Media

The mineral medium used for both the batch and continuous experiments and to store both the cell suspensions and beads was the same as that described by Martins dos Santos (1998). $(NH_4)_2SO_4$ and $KNO_2$ at a concentration of 10 and 5 mmol/l, respectively, were supplemented to all batch experiments, with exception of those containing co-immobilized nitrifiers and Anammox cells, to which only $(NH_4)_2SO_4$ was added at an end concentration of 10 mM. In the continuous experiment the medium contained $(NH_4)_2SO_4$ for the first 21 days and 15 mM thereafter. Furthermore, the medium was supplemented with 10 mM KCl so to maintain the stability of the gel beads.

### Immobilization procedure

*Nitrosomonas europaea* or the nitrifying sludge and the Anammox sludge were co-immobilized in a similar way as that described by Hunik *et al* (1994) for mixtures of nitrifying cells. Hence, a mixture of cell suspensions (or sludges) was mixed with a κ-carrageenan solution (Genugel X0828, A/S Kobenhavns Pektinfabrik, DK Lille Skensved) so that the final gel concentration was 2.5 % (w/v). The mixture (at 39 °C) was then extruded dropwise through a hollow needle (0.6 mm in diameter) into a cold 0.75 M KCl solution. Beads with a diameter of 4.5 ± 0.6 mm were obtained.

**Cultivation of suspended and immobilized cells**

***Batch experiments***

Removal of nitrogen compounds by co-immobilized *Nitrosomonas europaea* and Anammox was evaluated by cultivating these microorganisms in batch at 30°C, with gentle agitation (100rpm) in a shaker. The cells were cultivated both in suspension and immobilized in single gel beads, and both under anoxic and aerobic conditions. All experiments were done in quadruplicate. The experimental procedures and conditions were the same as those described elsewhere (Martins dos Santos *et al.*, 2001).

***Continuous experiments***

A continuous internal air-lift loop reactor was set up in a similar way as that earlier described (Martins dos Santos *et al.*, 1996b, 2001). The pH was set within the range 7.46 - 7.70 and the temperature was maintained at 30°C. The reactor had a working liquid volume of 483 ml of which approximately 20% were beads. The beads were the same as those used in the batch experiment. The retention time was 2.5 hours and was raised to 3.5 hours at day 34. Liquid samples were taken each day from the effluent and influent of the reactor and analyzed for nitrite, nitrate and ammonium. At the end of the experiments, part of the beads were transferred to serum flasks and cultivated in batch aerobically in medium that contained ammonium but no nitrite. The compositions of both the liquid and gas phase were monitored. At the end of the batch cultivation, the flasks were supplemented with fresh medium, were made oxygen and nitrogen free and were cultivated again for about three days. After that time, 5 mM of nitrite were injected into the flask, which was subsequently incubated for another 3 days.

**Analytical methods**

Ammonium, nitrite and nitrate concentrations in batch media and in the influent and effluent of the reactors were measured by colorimetry as described elsewhere (Hunik *et al.* 1994). Biomass concentrations were estimated from dry-weight measurements and biological oxygen monitoring as described earlier (Martins dos Santos *et al.*, 2001).

**Model development**

We have previously reported on the development and respective experimental validation of a dynamic model that simulates the behavior of autotrophic nitrifying and heterotrophic denitrifying cells co-immobilized in double-layer beads (Martins dos Santos *et al.*, 1996b, 2001). The model describes diffusion of components, substrate utilization and growth, all occurring simultaneously in the beads. Both internal and

external mass transfer resistance is accounted for. It predicts biomass and solute bulk concentrations, substrate consumption rates, product formation rates, and biomass and solute concentration profiles within the beads as function of time. Fluctuations in substrate load, dilution rates or mass transfer parameters can be accommodated as well. Intrinsic kinetic parameters of the microorganisms, internal and external mass transfer coefficients, initial conditions, bead concentration and particle diameters are the input parameters.

Table 1 - Model parameters

| Parameter | Value | Dimensions |
|---|---|---|
| $\mu_{max}^{Ns}$, $\mu_{max}^{Nb}$, $\mu_{max}^{Ax}$ | $1.52 \times 10^{-5}$; $1.0 \times 10^{-5}$; $8.2 \times 10^{-7}$ | $s^{-1}$ |
| $m^{Ns}$; $m^{Nb}$, $m^{Ax}$ | $5.44 \times 10^{-4}$; $2.2 \times 10^{-3}$; $5.44 \times 10^{-4}$ | mol .$kg^{-1}.s^{-1}$ |
| $Y_{NH_4}^{Ns}$; $Y_{O_2}^{Ns}$ | $2.16 \times 10^{-3}$; $1.57 \times 10^{-3}$ | kg.$mol^{-1}$ $NH_4$, $O_2$ |
| $Y_{NO_2}^{Nb}$; $Y_{NH_4}^{Ax}$ | $0.58 \times 10^{-3}$; $1.25 \times 10^{-3}$ | Kg.$mol^{-1}$ $NO_2$, $NH_4^+$ |
| $K_{O_2}^{Ns}$; $K_{NH_4}^{Ns}$ | $5.05 \times 10^{-3}$; 1.25 | $mol.m^{-3}$ |
| $K_{O_2}^{Nb}$; $K_{NO_2}^{Nb}$ | $17 \times 10^{-3}$; 0.36 | $mol.m^{-3}$ |
| $K_{NO2}^{Ax}$; $K_{NH4}^{Ax}$ | 0.83 ; 0.46 | $mol.m^{-3}$ |
| $X^{Ns}{}_{max}$; $X^{Nb}{}_{max}$; $X^{Ax}{}_{max}$ | 36.7; 11; 24 | $kg.m^{-3}$ |
| $K_{l,s}^{NH_4}$, $K_{l,s}^{NO_2}$ | $2.21 \times 10^{-5}$ | $m.s^{-1}$ |
| $K_{l,s}^{O_2}$ & $K_{l,s}^{N_2}$ | $2.63 \times 10^{-5}$ | $m.s^{-1}$ |
| $D_w^{NH_4}$ & $D_w^{NO_2}$ | $2.20 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $D_w^{N_2}$, $D_w^{O_2}$ | $2.83 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $D_{gel}^{NH_4}$ & $D_{gel}^{NO_2}$ | $1.78 \times 10^{-9}$ | $m^2.s^{-1}$ |
| $D_{gel}^{N_2}$, $D_{gel}^{O_2}$ | $2.30 \times 10^{-9}$ | $m^2.s^{-1}$ |

The model has a mechanistic basis as it is founded on general process equations and on the intrinsic kinetics of the cells. Therefore, we applied the model to the process described in this work by introducing the kinetic parameters of the Anammox and by changing the criteria for the choice of the "most limiting" substrate as described by Martins dos Santos *et al.* (1996b). According to this criterion, if two substrates are in theory limiting, the actual substrate conversion will be governed by the "most-limiting" substrate, which is the one with the lowest "Monod" term (Hunik *et al.*, 1994, Mankad & Nauman, 1992, dos Santos *et al.* 1996b). Anaerobic ammonium oxidation was

considered to proceed as proposed by van de Graaf *et al.* (1996) and Strous *et al.* (1998).

To possibly describe the interactions between nitrifiers and Anammox and between the nitrifying populations themselves (typically present in any nitrifying sludge such as that used here), the model was extended to three microbial species populations (*Nitrosomonas* spp., *Nitrobacter* spp. and Anammox), all interacting with each other. The parameters used were those described by Hunik *et al.* (1994) for *Nitrobacter agilis.* and *Nitrosomonas europaea.* The parameters for Anammox were taken partly from Van de Graaf *et al.* (1995,1996) and Strous *et al.* (1998) and partly from unpublished laboratory experiments. A list of all the parameters used in the model are presented in Table 1.

## RESULTS AND DISCUSSION

### Conversion of N-compounds by cells co-immobilized in single-layered gel beads

To verify whether the proposed system did indeed work, *Nitrosomonas europaea* was co-immobilized with Anammox sludge in single-layered gel beads and tested in batch, both under aerobic and anaerobic conditions, for their ability to remove ammonium and nitrite. High concentrations of both nitrifying cells and Anammox sludge were chosen so to have results within a reasonable time span.

Figure 1 shows the aerobic cultivation of such beads in a medium containing about 10 mM of ammonium and no nitrite. The concentration of ammonium decreased rapidly down to about 3 mM, with the greatest decrease at the start of the experiment (1a). Concomitantly, nitrogen gas accumulated up to about 2.5 $mmol.l^{-1}$, whereas the oxygen concentration decreased to negligible levels (not shown). The oxygen concentration did not increase above 1.5 mM even when larger amounts of oxygen were injected (not shown). This indicates that the *Nitrosomonas europaea* cells were very active and that they used up the oxygen as soon as it was available. The greatest changes in nitrogen and oxygen concentrations took place in the first 65 hours of the experiment. The amount of nitrite produced was minimal and temporary, indicating that the Anammox cells were quite active as well and that they consumed nitrite as soon as it was produced. A little nitrate was occasionally found as well as traces of $N_2O$. The nitrogen balances closed within an average of 5 % of the total.

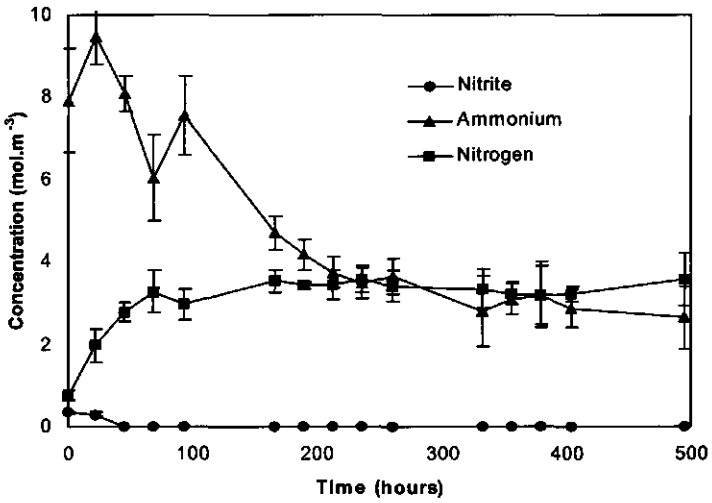


Figure 1 - Aerobic batch cultivation of *Nitrosomonas europaea* cells and Anammox sludge co-immobilized as a mixed culture in single-layered beads. Concentration of N-compounds in the liquid (ammonium and nitrite) and gas phase (nitrogen).

To verify whether the nitrogen resulted indeed of the Anammox activity, the beads were washed twice with phosphate buffer and cultivated for about 100 hours in the absence of oxygen and with ammonium at 10 mM. During this period, ammonium was not consumed nor nitrogen was produced indicating no ammonium-oxidizing activity.

This agrees with the findings of Van de Graaf *et al.* (1995,1996) and Strous *et al.* (1997a) who have clearly shown that the Anammox process occurs strictly under anaerobic conditions and require roughly equimolar amounts of nitrite and ammonium. Subsequently, the reaction flasks were supplemented with 5 mM nitrite. The nitrite concentration decreased rapidly to zero within about the next three days whereas nitrogen gas accumulated in the flask at a level that remained constant during the rest of the experiment (Figure 2). By injecting about 20% (v/v) oxygen in the flask, the remaining ammonium disappeared rapidly and nitrogen accumulated to a higher concentration.

Clearly, these results indicate that coupled autotrophic nitrification by nitrifying bacteria and anaerobic ammonia oxidation by the Anammox sludge took place in the beads. The rates attained were in the same range of those achieved in the system with *Nitrosomonas europaea* and Anammox immobilized in separate layers of double-layered beads described earlier (Martins dos Santos *et al*, 2001).

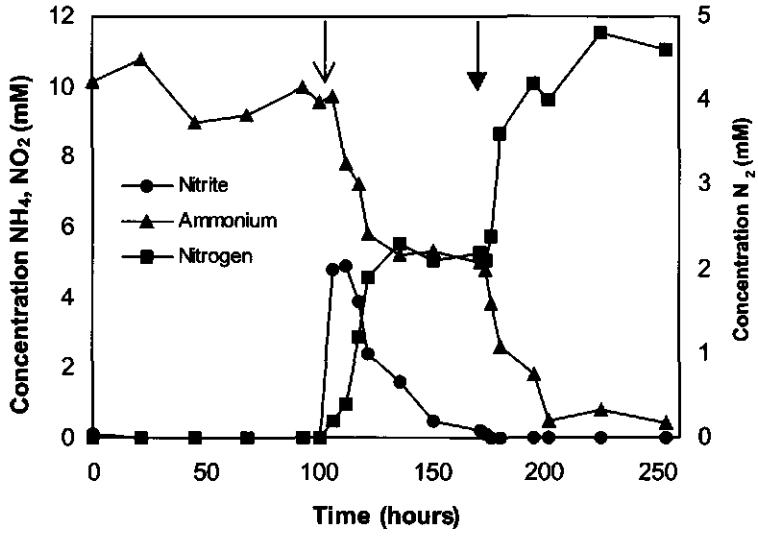


Figure 2 - Anaerobic batch cultivation of *Ns. europaea* cells and Anammox sludge co-immobilized as a mixed culture in single-layered beads. Concentration of N-compounds vs. time. Arrows indicate injections of nitrite (5 mM, open arrow) and oxygen (20% (v/v), closed arrow).

**Experimental design of the continuous process**

The results obtained in the batch experiments above were quite promising regarding the application of the proposed system for autotrophic nitrogen removal from wastewaters. Yet, a straightforward use of such beads in a continuous reactor does not guarantee a success because the both the operating and initial conditions may strongly influence the performance and effectiveness of the system as a whole. Indeed, two consecutive attempts of running a continuous reactor containing beads with co-immobilized nitrifiers and Anammox simply failed (results not shown). The failures were because ammonium was either fully converted to nitrite (one of the attempts) or even further to nitrate (the other attempt, in which a nitrifying sludge containing both *Nitrosomonas* spp. and *Nitrobacter* spp. was used instead of a pure culture of *Nitrosomonas europaea*). The reason for the first unsuccessful run was that *Ns. europaea* consumed most of the ammonium available in the reactor, preventing thereby the Anammox to oxidize ammonium together with the nitrite produced. In the second run, the *Nitrobacter* spp. present in the nitrifying sludge successfully outcompeted Anammox because it has both a higher affinity for nitrite and a much higher specific growth rate (see Table 1). In this run, Anammox was thus deprived of both nitrite and ammonium.

These failures were important in the sense that they laid open the problems and needs of the proposed system. It was hence clear that a careful design of the process was essential before the process could be implemented.

***Model results***

A rational design of the system proposed demands a thorough knowledge of the mechanisms underlying the processes involved. Therefore, a dynamic model, previously developed and validated for both nitrification alone (Hunik *et al.*, 1994) and simultaneous autotrophic nitrification and heterotrophic denitrification in magic beads (Martins dos Santos *et al.*, 1996b), was adapted to include the sludge responsible for the Anammox process.

As a first approach to test the validity of the model for this application, previously published results reporting autotrophic growth of Anammox microorganisms in a fluidized bed reactor (van de Graaf *et al.*, 1996) were compared with the corresponding predictions yielded by the model. For modeling purposes, the sand supports covered by a biofilm reported by van de Graaf and co-autors were assumed to be equivalent to single gel beads of the same diameter. Initial biomass concentrations were estimated from the biomass concentrations (in mg $VSS/g_{carrier}$), reported by Mulder *et al* (1995) taking into account a volumic mass of sand of 2.6 $kg.l^{-1}$ and that the initial sludge contained only 16% of the cells thought to be responsible for the Anammox process (van de Graaf *et al.*, 1996). The experimental results earlier reported are presented in Figure 3a, whereas Figure 3b shows the corresponding model predictions. Clearly, the trend given by the model predictions agrees well with the experimental results. The changes in the operating conditions (stepwise increase in the influent substrate concentrations) were appropriately accounted for as well. Subsequently, the model was used to predict whether or not nitrifying cells and Anammox sludge would "cooperate" if co-immobilized as a mixed culture immobilized in single-layered gel beads.

Simulations with *Ns. europaea* co-immobilized with Anammox sludge showed that, for each particular set of conditions (for instance, for a given bead diameter), if there is a sufficient ammonium load (so that there is $NH_4^+$ left for the Anammox) and if both the initial Anammox-cells concentration and the initial ratio Anammox:nitrifiers is high enough (typically 100 to 1000), ammonium can be successfully converted into nitrogen at high rates in a continuous system with single-layered gel beads.

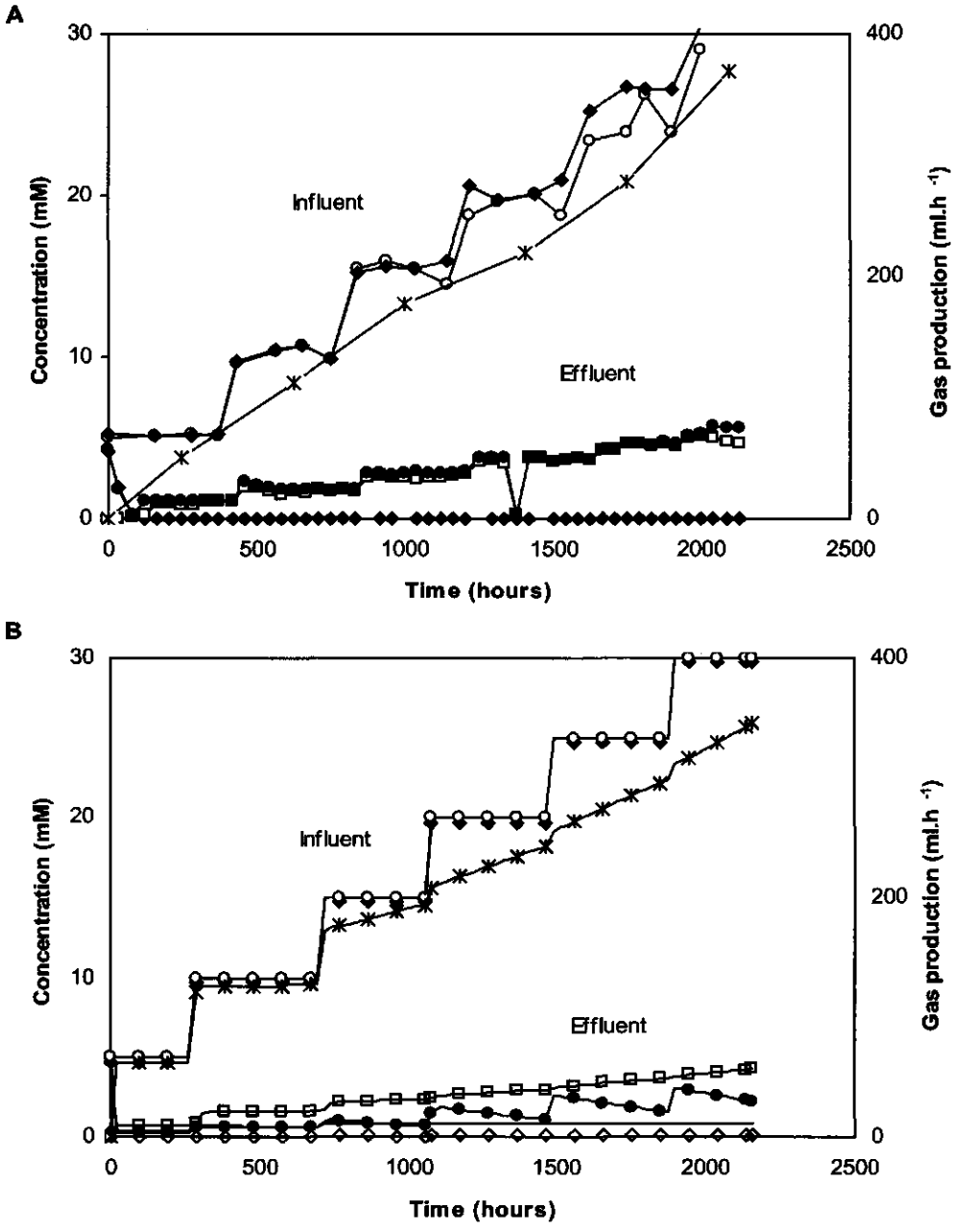


Figure 3 - Continuous operation of a fluidized bed reactor with Anammox biofilms as described by van de Graaf *et al.*, 1996: a) measured bulk concentrations (Figure adapted from van de Graaf *et al.*, 1996); b) model predictions. Influent: ◆, $NH_4$, ○ $NO_2^-$; Effluent: ●, $NH_4^-$, ✧, $NO_2^-$; □, $NO_3^-$; ✳, gas production.

**Continuous nitrogen removal**

Single-layered beads were newly made and transferred to a continuously operated air-lift loop reactor. A nitrifying sludge containing about 98% *Nitrosomonas* spp. and 2% *Nitrobacter* spp. was used instead of a pure culture of *Ns. europaea* because such sludge approaches better the common wastewater-treatment practices. The results are shown in Figure 4a.

The bulk ammonia concentration decreased rapidly and that was accompanied by a corresponding increase in nitrite, which indicated active metabolism of *Nitrosomonas* spp. After day 10, nitrate began to accumulate whereas nitrite disappeared. Since there was practically no ammonium left in the bulk, this increase in nitrate and corresponding decrease in nitrite is likely to be explained by growth of *Nitrobacter* spp. rather than by intense Anammox activity. A failure in the pH-control on day 15 resulted in a decrease of pH to about 6, which led to a sharp but temporary decrease in the nitrifying activity. This activity seemed to be recovered by day 20.

To prevent ammonium limitation of the Anammox sludge and thus possible overgrowth by *Nitrobacter* spp. cells, the influent ammonium concentration was raised to about 16 mM at day 24. The Anammox activity increased slowly and reached significant levels (0.4-0.6 kgN kg-$N.m^{-3}_{reactor}$) only after this increase in the influent ammonium concentration. A decrease in the ammonium concentration and an increase in nitrite followed the rise of the retention time from 2.5 to 3.5 hours on day 34. The nitrogen removal rates, however, increased at approximately the same speed as before the change. The overall ammonium conversion rates reached about 1.2-1.4 kg-$N.m^{-3}_{reactor}.day^{-1}$ whereas those of nitrogen formation reached a maximum of 0.6-0.8 kg-$N.m^{-3}_{reactor}.day^{-1}$ (3-4 kg-$N.m^{-3}_{gel}.day^{-1}$). After the ammonium concentration had decreased to about 1-0.5 mM nitrate began to accumulate at higher rates indicating that the *Nitrobacter* spp. were again competing successfully for nitrite with the Anammox cells.

The specific removal rates achieved were comparable to those of nitrification or denitrification alone in reactor-systems based either on artificially entrapped microorganisms (Tanaka *et al.*, 1991, Wijffels *et al.* 1989, 1990, 1991, Nilsson *et al.* 1982, Kokufuta *et al.*, 1989) or naturally attached biofilms (Tijhuis *et al*, 1994, Tanaka & Dunn, 1982). The rates were also similar to those earlier obtained by van de Graaf *et al* (1996), Strous *et al.* (1997b) and Mulder *et al.* (1995) for the anaerobic oxidation of ammonium.

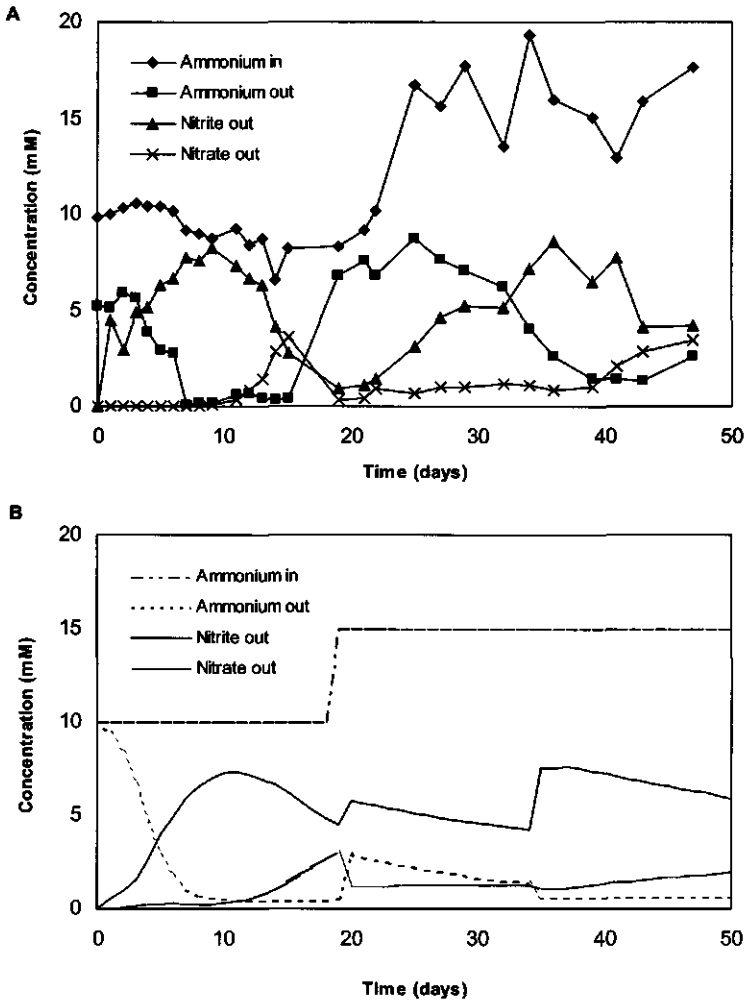


Figure 4 - Continuous cultivation of *Nitrosomonas* spp., *Nitrobacter* spp. and Anammox sludge co-immobilized as a mixed culture in single-layered beads: a) measured bulk concentrations; b) model predictions.

Figure 4b shows the bulk concentrations predicted by the model for the experimental conditions of the continuous run. As it is difficult to account for the effects of a sudden pH change, the days after the shock were not considered in the simulation and thus the ammonium step was applied at day 19 instead of at day 24. Despite of some discrepancies, the model predicted properly the trends in the system's behavior and took

well into account the changes in the operation conditions. The model also described well the nitrite consumption and corresponding nitrate production by *Nitrobacter* spp. cells.

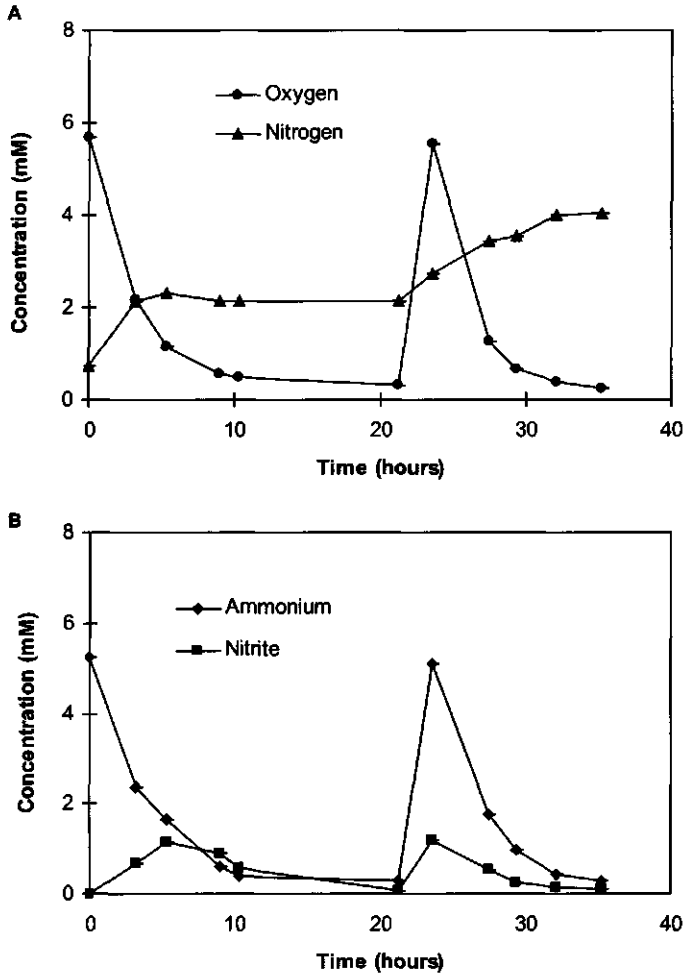


Figure 5 - Aerobic batch cultivation of nitrifying and Anammox sludge co-immobilized as a mixed culture in single-layered beads (From Figure 4a). Concentration of N-compounds in: a) liquid phase; b) gas phase.

After 47 days, the experiment was stopped and the beads were transferred to nitrogen- and oxygen-free flasks that were then incubated in batch after being injected with oxygen and ammonium. Practically all ammonium (Figure 5a) and oxygen (Figure 5b) were depleted after about 10-11 hours. Within the same period, nitrite was formed and

subsequently consumed (5a) whereas nitrogen up to about 2 mmol.$l^{-1}$ accumulated in the headspace (5b). Injection of oxygen and ammonium led to a similar response. These results indicate that the "missing N" in the N-balance of the continuous run was indeed likely to be nitrogen produced by Anammox. To confirm that, the beads were incubated with fresh medium under anaerobic conditions. Only a little ammonia was removed, possibly due to some nitrite still present within the beads. After injection of nitrite, both ammonia consumption and nitrogen production increased steeply (results not shown).

***Anaerobic/aerobic zones***

That a fundamentally aerobic process (nitrification) and a strictly anaerobic one (Anammox) can take place simultaneously within a bead can be attributed to the establishment of aerobic and anaerobic regions across the particles. We have shown that this is indeed so by measuring the oxygen, nitrite and ammonium concentration profiles across a double-layered gel matrix with co-immobilized autotrophic nitrifiers and heterotrophic denitrifying cells (Martins dos Santos *et al.*, 1999). In that system, physical separation of the two microbial populations in two gel layers was necessary to prevent overgrowth of the nitrifying population by the faster-growing heterotrophic facultative denitrifiers. In the system proposed in this paper, however, since the microorganisms involved have very different oxygen requirements, a spontaneous "habitat segregation" of the microbial populations would be expected at the medium-long term if the cells would be simply co-immobilized as a mixed culture in single gel beads (Kurosawa and Tanaka, 1990). Due to fast oxygen uptake by the nitrifying cells, oxygen gradients within the beads developed which in turn led to the establishment of biomass gradients across the particle. The nitrifying cells would have then concentrated in a thin layer underneath the bead surface whereas the Anammox population would have preferred zones depleted of oxygen. This behavior is clearly illustrated by the model output on Figure 6. In 6a, the distribution of the three populations is shown for day 19, when the system is fully active and just prior the step in influent ammonium.

The nitrifying populations accumulated mainly at the outermost bead layers whereas Anammox grew in the oxygen-free core. This biomass distribution matches well the substrate profiles across the particle (Figure 6b). "Habitat segregation" between nitrifying and ammonium oxidizing cells is clear and entirely coupled to the oxygen distribution. Such a distribution of the nitrifying populations has been repeatedly measured in gel beads by different methods (Hunik *et al.*, 1994, Wijffels *et al.*, 1991, Leenen *et al.*, 1996) and was shown to be well described by dynamic models similar to that described here.

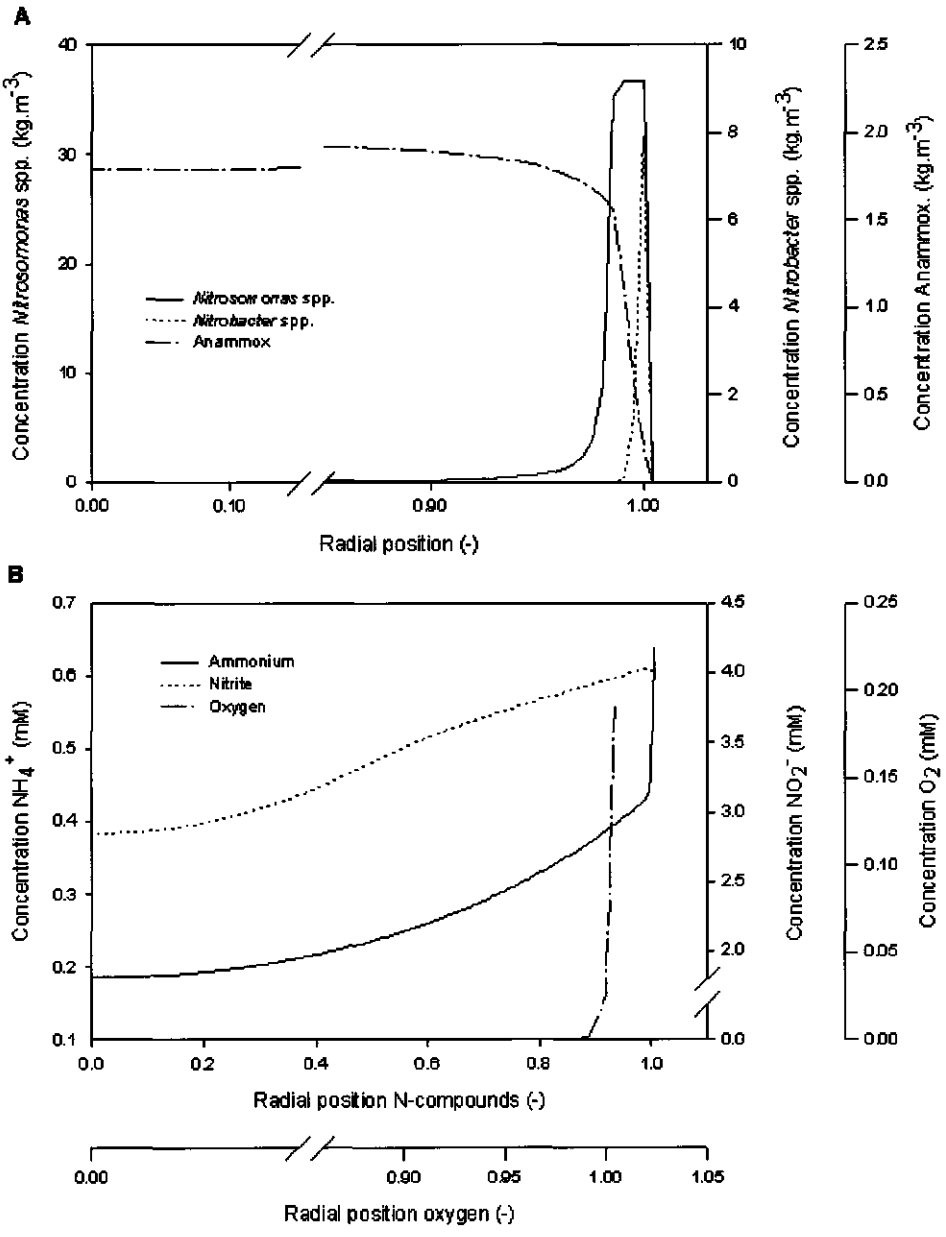


Figure 6 – Simulated biomass (A) and substrate (B) profiles across the single-layered gel beads for day 19 of the experimental run (cf. Figure 4). Note the different scales both on the Y and X-axis, which were set to facilitate visual analysis. Total bead radius: 2.4 mm.

**Interactions between the cell populations**

Although ammonium was effectively removed in the continuous experiment with single-layered gel beads, it is clear from Figure 4 that the microorganisms responsible for the Anammox process had to compete fiercely both for ammonium and nitrite, respectively with *Nitrosomonas* spp. and *Nitrobacter* spp. The Anammox microorganisms depend on the aerobic activity of *Nitrosomonas* spp., which provide them with both nitrite and the anaerobic conditions they need. However, the Anammox sludge is as well dependent on the ammonium that is left from the nitrifying activity. If there is little ammonium available the Anammox activity is strongly limited. Under these conditions, *Nitrobacter* spp. (even if immobilized at low initial concentrations) can compete successfully for nitrite. Indeed, in the continuous experiment of Figure 4, the accumulation of nitrate following depletion of ammonium between days 6 and 20 and after day 40-41 show that the *Nitrobacter* spp. cells were competing successfully for nitrite with the Anammox cells. Between day 24 and day 41, a period of relative ammonium "abundance", the relatively low concentrations of nitrate produced (of the order of those that would be expected from Anammox activity) indicate a reduced activity of *Nitrobacter* spp.. Although *Nitrobacter* spp. cells have higher affinity for nitrite and a maximum specific growth rate about one-and-a-half order of magnitude higher than that of Anammox (so that in theory they would easily overgrow the Anammox), they also have to compete for oxygen with *Nitrosomonas* spp., which have higher affinity for oxygen (Hunik *et al.*, 1994) and were immobilized in much higher concentrations. Due to that, the *Nitrobacter* cells were also severely limited in their activity and could thus be outcompeted by Anammox if ammonium was readily available. These interactions between the three populations and their competition for ammonium, nitrite and oxygen (competition between *Nitrobacter* and *Nitrosomonas*, inihbition of Anammox activity) were well described by the model, as clearly reflected in Figures 4b and 6. The model provides thus a good and realistic view of processes involved. These results showed also that the competitions for substrates by the populations involved can be decisive in determining the effectiveness of the system here proposed. A careful design and adequate control of the operating conditions are therefore important to guarantee a stable conversion of ammonium into nitrogen.

**Process design**

Compared to double-layer gel beads, single-layered beads are much simpler to make and, since the microbial populations are co-immobilized as mixed cultures, both the ammonium and nitrite necessary for the Anammox would be, in theory, more readily

available. Due to that, it would be expected that single-layered bead systems would reach higher biodegradation rates than those that make use of double-layer beads. Although this was indeed observed in the continuous experiments here described, a direct, quantitative comparison cannot be made because the operating conditions (as well as the initial biomass concentrations and bead diameter) were different for both.

A way to compare the performance and effectiveness of both systems is by using the mathematical model proposed here as it proved to describe well the behavior and the interactions between the microorganisms involved (see also Martins dos Santos *et al*, 1996b and Hunik *et al*, 1994). Furthermore, since the model is a dynamic one, changes in the operating conditions (common in the wastewater-treatment practice) can be appropriately accounted for. Besides the insight into the complex mechanisms underlying the processes involved it gives, the value of such a mechanistic model is the possibility to use it, with care, for predictions beyond the experimental boundaries (Beck, 1991), which allow us to design and improve the system's performance and robustness.

In Figure 7a, the continuous experiment described above is simulated for 200 days. Besides the already mentioned concentration step at day 19 and a decrease in the dilution rate at day 34 (Figure 7b), in this simulation the ammonium concentration was increased in steps of 5 mM at the days 50, 60, 80, 100, 120 and 150. A simulation with double-layer beads, under the same operating conditions and with the same initial parameters as those with single-layered beads is presented in Figure 7b. Clearly, the system with single-layered beads reaches higher biodegradation rates than the double-layered system. In the double-layer bead reactor, only about 62% of the ammonium is converted (50% overall nitrogen removal) at the end of the simulation with an influent load of 4.5 kg-N.$m^{-3}_{reactor}$.$d^{-1}$. In contrast, in the single-beads system, 91% of the ammonium is converted and 86% of the total nitrogen is removed (Figure 7a). This is likely due to the much shorter distances for the transport of substrates to the microorganisms responsible for the Anammox process as compared to doubled-systems. As opposed to suspended-cell systems, most of which are exclusively dependent on the growth of biomass, immobilized-cell systems are largely limited by mass transport and relatively less influenced by kinetics (de Gooijer *et al.* 1991, de Backer *et al.*, 1996). Indeed, the simulations of Figures 7a and 7b were done for total radius of 2.4 mm. In 7b, the inner and outer layers had a radius and a thickness of 1.2 mm, respectively. Anammox cells are thus strongly limited by diffusion because they are far from the zone where ammonium and nitrite are being consumed and produced, respectively.

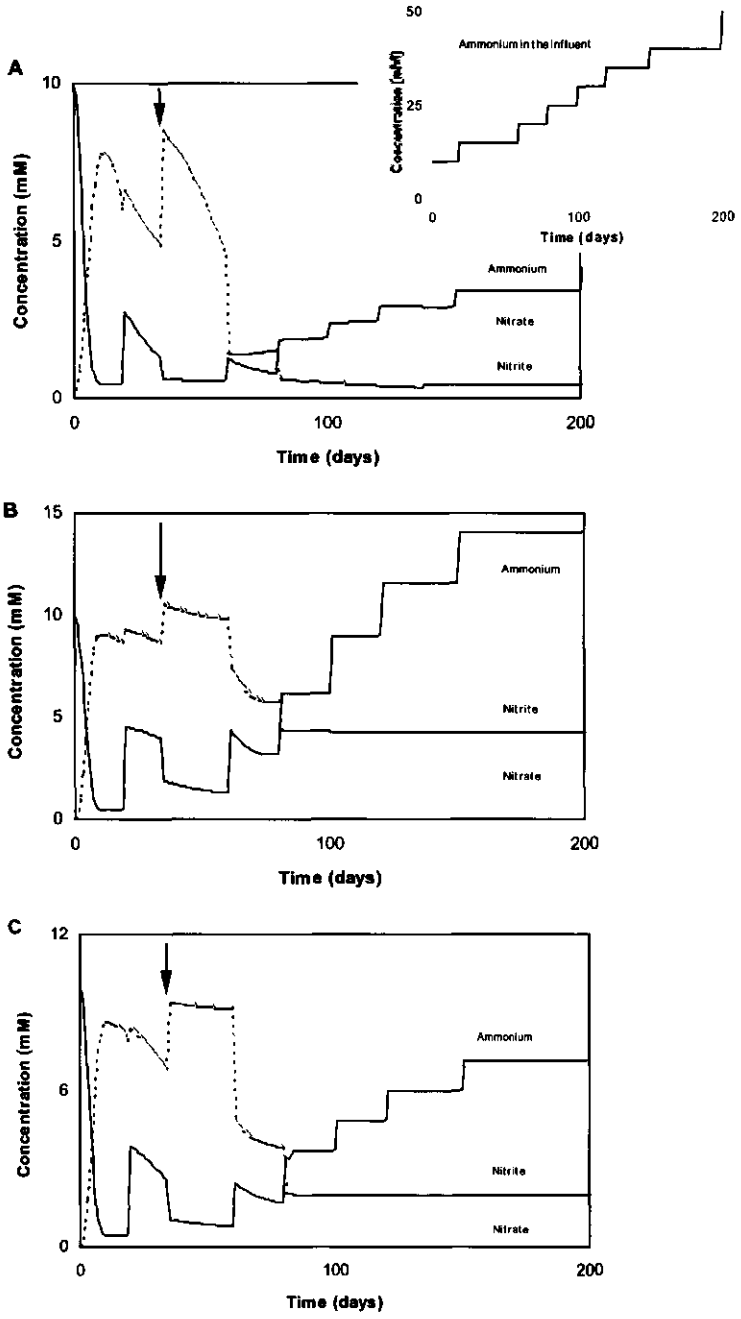


Figure 7 - Simulations of the influent (insert in A) and effluent bulk concentrations of N-compounds from the continuous cultivation of *Nitrosomonas* spp. *Nitrobacter* spp.and Anammox co-immobilized in: a) single-layered gel beads, total radius: 2.4 mm; b) double-layered gel beads; inner radius: 1.2 mm; thickness outer layer: 1.2 mm; c) double-layered beads; inner radius: 1.8 mm; external layer: 0.6 mm. Arrows indicate change in dilution rate.

A simulation done with double-beads having an inner radius of 1.8 mm and a external-layer thickness of 0.6 mm shows that twice as much ammonium per unit time per day is removed (Figure 7c). In this situation, the Anammox cells are much closer to the nitrifying layer so that they are less limited by diffusion. Moreover, since Anammox cells grow very slowly and their affinity constants for substrate are relatively low, the biomass profiles established across the bead are not very steep within the time span of the simulations (see e.g. Figure 6a). This means thus that most of the biomass immobilized in the bead is important for conversion and not just a thin layer of cells such as it happens with *Nitrosomonas*. Therefore, beads with larger inner diameter contain more biomass and convert more.

A simulation in which the inner radius and thickness were 2.1 and 0.3 mm, respectively, showed conversion rates comparable to those of the simulation with single-layered beads in 7a).

***Process improvement***

Although ammonium was removed at considerable high rates, there was still a considerable part of it that was only converted into nitrite. As discussed above, this happened likely because Anammox was limited by the ammonium available. Under these conditions, it seems desirable to somewhat limit the activity of the nitrifying microorganisms so that more ammonium is available for anaerobic ammonium oxidation. The model proposed can be a useful tool to evaluate this hypothesis. For instance the model predicts that, compared with Figure 4b, twice as much ammonium is converted into nitrogen by running the reactor at an oxygen bulk concentration that is 60% of oxygen air saturation at 30°C instead of the 80% of Figure 4b. By lowering the oxygen bulk concentration, much less nitrifying biomass is able to oxidize ammonium (see profiles in Figure 6), which becomes thus available for Anammox. However, by lowering the bulk oxygen concentration to 40% air saturation, about half of the total ammonium remains unconverted because now not enough nitrite is produced for optimal anaerobic ammonium oxidation. These results suggest that regulation of the oxygen concentration in the bulk may thus provide a way to optimize the overall nitrogen removal rates for every set of operating conditions. Furthermore, reduction of the oxygen concentration in ° the bulk may be also a way of minimizing undesirable competition by *Nitrobacter* spp. Indeed, the model predicts that there is practically no nitrite-oxidizing activity by *Nitrobacter* at 60% oxygen saturation in the bulk (instead of 80% in Figure 4b). Under these conditions (in which ammonium is present at non-limiting concentrations for *Nitrosomonas*), most of the oxygen available is consumed by

the *Nitrosomonas* cells. These cells have a maximum specific growth rate and an affinity for oxygen that is about one-and-a-half and three times higher, respectively, than that of *Nitrobacter* (Hunik, *et al.*, 1994). These predictions agree with the experimental results of Tanaka and Dunn (1981, 1982) and Garrido-Fernandez *et al.* (1996), which showed that the nitrite-oxidation rates of a nitrifying biofilm decreased more than the ammonium-oxidation rates for decreasing bulk oxygen concentrations. Both groups attributed these results to the difference in the kinetic parameters between *Nitrosomonas* and *Nitrobacter* cells. Clearly, thus, for each set of conditions the whole process should be controlled by the correct supply of oxygen and ammonium.

This brief analysis shows that a careful design of the reactor system is required to achieve the most optimal conditions for effective and efficient nitrogen removal. It demonstrates also that a dynamic model such as that proposed here is an useful tool to design and to predict the outcome of a process at various experimental conditions. The bottlenecks can be easily identified and the factors that influence the most the system's behavior and that may thus limit its biodegradation performance can be studied.

**Perspectives**

The nitrogen removal system described here is based on autotrophic, slow-growing cells having relatively low yields of biomass. These characteristics have important advantages for its application in wastewater treatment processes. Besides its compactness and the considerable savings both in organic compounds (100% compared to heterotrophic denitrification) and oxygen (50% by avoiding intermediate nitrate formation and aerobic oxidation of half of the ammonium), the system leads to very low sludge production, which is often an important problem in conventional denitrification plants (Jetten *et al.*, 1998. Additionally, the acid produced in the nitrification step by nitrifiers is partially compensated by the base resulting from the anaerobic oxidation of ammonium, which facilitates greatly the pH control in such a reactor. By being an autotrophic process, the system can be uncoupled from COD removal, which strongly reduces the risks of overgrowth by heterotrophic microorganisms. Providing that an appropriate carrier material is chosen and that the system is well designed, the process proposed here is a very interesting option for the removal of nitrogen from wastewaters, either as a post-treatment step or directly from streams with low organic-carbon content.

## ACKNOWLEDGEMENTS

Financial support by the Junta Nacional de Investigação Científica e Tecnológica (JNICT), Portugal is gratefully acknowledged.

## REFERENCES

Beck, M.B. 1991. Principles of modeling. Water Sci. Technol. 24:1-8

de Backer, L., R.G. Willaert and G. V. Baron. 1996. Modeling of immobilized bioprocesses. pp. 47-66. *In:* Baron G.V. and L. de Backer (eds). Immobilized living cell systems - modeling and experimental methods. Jonh Wiley & Sons. New York, USA.

de Gooijer de C.D., Wijffels R.H., Tramper J. 1991. Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in carrageenan: part 1. Dynamic modeling. Biotechnol. Bioeng. 38:224-231.

Garrido-Fernadez, J.M., W.A. J. van Benthum, M.C.M. van Loosdrecht and J.J. Heijnen. 1996. Influence of dissolved oxygen concentration on nitrite accumulation in a biofilm airlift suspension reactor. Biotechnol. Bioeng. 53:168-178

Hunik J.H., Bos C.G., Van Den Hoogen P., De Gooijer C.D., Tramper J. 1994a. Co-immobilized *Nitrosomonas euroapaea* and *Nitrobacter agilis* cells: validation of a dynamic model for simultaneous substrate conversion and growth in k-carrageenan gel beads. Biotechnol. Bioeng., 43:1153-1163.

Jetten, M.S.M., Horn S.V.M.C.M. van Loosdrecht, L.A.1997. Towards a more sustainable municipal wastewater treatment system. Wat. Sci.Technol.35(9):171-180

Kokufuta E., M. Shimoshashi and I Nakamura. 1987. Continuous column denitrification using polyelectrolyte complex-entrapped *Paracoccus denitrificans* cells. J. Ferment.Technol. 65:359-361

Kurosawa H. and H. Tanaka. 1990. Advances in immobilized cell culture: development of a co-immobilized mixed culture system with aerobic and anaerobic microorganisms. Process Bioche. Int. 25: 189-196.

Leenen, E.J.T.M., Boogert AA, van Lammeren AAM, Tramper, J., Wijffels, R.H. 1997. Dynamics of artificially immobilized *Nitrosomona europaea* : effect of biomass decay. Biotechnol Bioeng. 55:630-641

Mankad T. and E.B. Nauman. 1992. Modeling of microbial growth under dual limitations. *Chem. Eng. J.* 48:B9-B11.

Martins dos Santos V.A.P.M., M. Bruijnse, Tramper J., Wijffels R.H. 1996a. The Magic-Bead concept: an integrated approach for nitrogen removal. Appl. Microbiol. Biotechnol. 45:447-453.

Martins dos Santos V.A.P.M., J.L. Marchal, J. Tramper J, R.H. Wijffels. 1996b. Modeling and evaluation of an integrated nitrogen removal with co-immobilized micro-organisms in double-layer gel beads. Biotechnol. Progress. 12: 240-248.

Martins dos Santos V.A.P.M., Tramper J., Wijffels R.H. 2001. Substrate and product profiles across double-layer gel geads: modelling and experimental evaluation. Submitted to for publication.

Mulder, A., A.A. van de Graaf, L.A. Robertson and J.G. Kuenen. 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol. Ecol. 16:177-184.

Nilsson I. and S. Ohlson. 1982. Columnar denitrification of water by immobilized cells. Appl. Microbiol Biotechnol. 14: 86-90.

Strous, M., E. van Gerven, J. G. Kuenen and M.S.M. Jetten. 1997a. Effect of aerobic and microaerobic conditions on anaerobic ammonium-oxidizing (Anammox) sludge. Appl. Environ. Microbiol. 63:2446-2448.

Strous, M., E. van Gerven, P. Zheng, J. G. Kuenen and M.S.M. Jetten. 1997b. Ammonium removal from concentrated waste streams with the anaerobic ammonium oxidation (Anammox) process in different reactor configurations. Wat. Res. 31:1955-1962.

Strous, M. M., E. van Gerven, J. G. Kuenen and M.S.M. Jetten. 1998. The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. Appl. Microbiol. Biotechnol. 50:589-596

Strous, M.,Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., van de Pas-Schoonen, K., Webb, R., Kuenen, J.G. and M.J.M Jetten. 1999. Missing lithotroph identified as new plactomycete. Nature 400,446-449

Tanaka H. and I. J. Dunn. 1981. Kinetics of nitrification using a fluidized sand bed reactor with attached growth. Biotechnol. Bioeng. 23:1683-1702.

Tanaka H. and I. J. Dunn. 1982. Kinetics of biofilm nitrification. Biotechnol. Bioeng. 24:669-689.

Tanaka K., T. Kimata, S. Harada, Y. Fujii, T. Mizuguchi, N. Mori and H. Emori. 1991. Development of new nitrogen removal systems using nitrifying bacteria immobilized in synthetic resin pellets. Water Sci. Technol. 23:681-690.

Tijhuis L. J.L. Huisman, H.D. Hekkelman, M.C.M. van Loosdrecht and J.J. Heijnen. 1994. Formation of nitrifying biofilms on small suspended particles in airlift reactors. Biotechnol. Bioeng. 47:585-595.

van de Graaf, A.A. A. Mulder, P. de Bruijn, M.S.M. Jetten, L.A. Robertson and J.G. Kuenen. 1995. Anaerobic ammonium oxidation is a biologically-mediated process. Appl. Environm. Microbiol. 61:1246-1251.

van de Graaf, A.A. A. Mulder, P. de Bruijn, M.S.M. Jetten, L.A. Robertson and J.G. Kuenen. 1996. Autotrophic growth of anaerobic ammonium oxidizing microorganisms in a fluidized bed reactor. Microbiology. 142:2187-2196.

Wijffels R.H. and J. Tramper. 1989. Performance of growing *Nitrosomonas europaea* cells immobilized in carrageenan. Appl. Microbiol. Biotechnol. 32: 108-112.

Wijffels R.H., De Gooijer C.D., Kortekaas S., Tramper J. 1991. Growth and substrate consumption of *Nitrobacter agilis* cells immobilized in κ-carrageenan. Part 2: Model evaluation. Biotechnol. Bioeng. 38: 232-240

Wijffels R.H., Schukking A, and J. Tramper. 1990. Characterization of a denitrifying bacterium immobilized in κ-carrageenan, Applied Microbiol. Biotechnol. **34**:399-403

This chapter has been submitted for publication by Martins dos Santos VAP, Tramper J, Wijffels RH.

## CHAPTER 12

# Towards an Integrated Approach for Oxidative and Reductive Biodegradation Processes

**Abstract**

Complete degradation of many pollutants requires sequenced anaerobic-aerobic biotreatment steps. Many compounds that are difficult to degrade aerobically are readily biotransformed anaerobically. The products of anaerobic biotransformation, however, will frequently resist to further mineralization; yet, they will be good substrates for aerobic biodegradation. Examples of this are the sequential biodegradation of highly chlorinated aromatics and aliphatics, azo-dyes, TNT, nitrogen compounds, and pesticides such as DDT, HCH's or methoxychlor. In waste- and groundwater treatment, these sequenced biotransformations are commonly achieved either by using aerobic and anaerobic (anoxic) reactors in series or by alternating periods of aerobiosis and anaerobiosis in a treatment unit. Ideally, however, these biodegradation processes would take place in a single, compact continuous-reactor system under carefully controlled conditions. This paper addresses the possibilities of integrating oxidative and reductive complementary biodegradation processes in compact systems by using co-immobilized mixed-culture systems. Relying on this view, perspectives on the biodegradation of recalcitrant compounds and bioremediation of contaminated sites are discussed.

## INTRODUCTION

Generally speaking, it could be said that if one waits long enough almost every natural compound, irrespective of its molecular weight or structural complexity, may be ultimately degraded by a given species or group of species in some particular environment. Indeed, the microbial flora on earth is so amazingly diverse, versatile and ubiquitous that about every "natural" pollutant can be, in one way or another, transformed and integrated into the natural biogeochemical cycles. The same holds for many synthetic compounds, which are generally funneled into the natural metabolic cycles by means of enzyme sets able to recognize synthetic structures resembling their natural substrates. An example of this is the metabolism of many industrially-produced aromatic compounds (in particular those structurally related to natural aromatics, such as toluenes and derivatives), which are transformed into (di)hydroxy intermediates (such as catechols) and thus channeled to central pathways like the Krebs cycle or the keto-adipatic pathway.

### *Xenobiotics*

Certain substituents, however, confer a rather recalcitrant character to synthetic compounds, which are then named xenobiotics (i.e. of antropogenic origin). Indeed, substituents (xenophors) such as halogen, sulfo-, azo- or nitrogroups, which have a very electron-withdrawing character and that are normally not present in natural environments, generate an electron deficiency in the whole molecule and make it resistant to microbial oxidation. Such compounds, and in particular those bearing multiple "xeno" substituents (xenophors) are quite recalcitrant and do not easily undergo (if at all) biodegradation under aerobic conditions.

Yet, slowly, more and more studies have been reporting that such multi-substituted xenobiotics may be partly biotransformed by anaerobic microorganisms through reductive attack (Knackmuss, 1996, Field *et al.*, 1995,etc.). Examples of this are the reductive dechlorination of polychlorinated aromatics and aliphatics (e.g. PCB's, HCH's, PCE'e), the reductive elimination of the nitro groups from polynitroaromatics (e.g. TNT, RDX), desulfonation of sulfonated compounds (e..g. detergents, pesticides or dyes) or the reductive cleavage of azo bonds (e.g. azo-dyes). However, only very rarely have these compounds been reported to be fully demineralized or at least converted into environmentally acceptable compounds. [There are, yet, already few honorable exceptions such as that of the complete anaerobic mineralization of Azodisalicylate reported by Razo-Flores *et al.* (1999) or the reductive dechlorination of PCE down to ethylene reported by Maymó-Gatell *et al.* (1997)].

Most generally, these xenobiotics are simply transformed into less substituted compounds that either remain unattacked, polymerize or just bind to soil particles. In many instances, these anaerobically dead-end metabolites could be, in principle, further mineralized under aerobic conditions because the part of the electron-withdrawing groups causing aerobic recalcitrance have been removed. This has been demonstrated manifold for a wide range of compounds and under a broad set of conditions (see Field *et al.* 1995 and Zitomer & Speece, 1993, for thorough reviews). Yet, and since quite often the right environmental conditions for the full degradation of such products of anaerobic transformation are not available at the site, these metabolites tend to remain there simply as dead-end products. Such metabolites (which often result from the misrouting of a non-productive metabolism) are sometimes even more hazardous than their precursors (e..g. azoxyamines and azoxydimers as dead-end products of TNT degradation or vinyl chroride (VC) from PCE's dechlorination) and may spread in subsurface soils, groundwater or even air.

***Waiting too long***

Hence, in many cases, "wait long enough" for the natural bioelimination of such "difficult" compounds may mean indeed waiting quite a long time. For instance, recent estimates indicate that it would take about 20 000 years before a single, natural microorganism could evolve so that it would be able to fully degrade some highly chlorinated dibenzodioxins, which are potent carcinogens (Muller, 1992). The situation becomes even more complicated if one considers that most contaminated streams of both industrial and agricultural origin are made up of complex mixtures of different compounds, of which many are themselves xenobiotic (e.g. wastestreams from textile industries, made up of quite complex mixtures of azo and sulfonated dyes). In addition, such compounds are not only intrinsically recalcitrant to microbial attack but are also frequently toxic at relatively low concentrations, and are often poorly water soluble, which seriously decreases their bioavailability towards microbial degradation. Finally, and besides the relatively sudden introduction of xenobiotics into the environment, even the massive relocation of natural materials to different environmental compartments (e.g. urban sewage into the rivers) may simply overwhelm the self-cleaning capacity of the recipient ecosystems.

**Integrating oxidative and reductive systems**

These series of facts clearly indicate that, in many cases, we have to help nature a bit. The ways of doing it have formed the basis of Environmental Biotechnology since its

beginning, by exploiting both the capacities of existing microorganisms, by genetically improving them and by looking for adequate technological solutions. Despite the many and important progresses made, however, handling contaminants such as those mentioned above, which would require both oxidative and reductive steps for their full transformation, is still largely an ill-resolved issue mainly due to the opposite oxygen requirements of both processes. In many instances, this need of using sequenced anaerobic-aerobic strategies for complete mineralization is even not clearly understood. Slowly, however, such needs have become clearer and more and more processes following sequenced strategies have been developed (Zitomer & Speece, 1993).

By far, the most common approach for implementing such strategies, for instance in wastewater treatment (which is the field where this rationale has been used for longer), is by coupling series of aerobic, anoxic and/or anaerobic reactors in different combinations within a given treatment plant [a typical, well established example is the removal of nitrogen, BOD and phosphorus by active sludge in sewage treatment plants]. Another common strategy is to apply, alternatively, aerobic and anoxic conditions to the same environmental compartment (a reactor, a soil or an aquifer). These general approaches (which have many possible variations according the materials to be handled) often work well and they are sometimes even the best or only alternatives available. Yet, ideally one would prefer to integrate both oxidative and reductive processes within a single compact, system as, in many cases and as we will see later on, the advantages of such an integrated system would be clearly greater than the mere sum of the individual advantages.

***The best of two worlds in a small particle***

As Field and colleagues noted (1995), the consortia responsible for anaerobic/anoxic biotransformation and those for subsequent aerobic mineralization need not to be necessarily separated in different reactors or distinct periods of operation. Indeed, evidence in literature indicates that anaerobic and aerobic consortia can occur side by side. For instance, Gerritse and colleagues (1993, 1990) have demonstrated that aerobic and anaerobic microorganisms may happily coexist in chemostats under carefully controlled aeration. In their studies, facultative anaerobes depleted the bulk from oxygen and allowed thereby the stricter anaerobes to thrive well.

Strict anaerobiosis can also occur in anaerobic niches inside biofilms and aggregates. A striking example is that reported by Kato *et al.* (1993), which have shown that methanogenic bacteria (strict anaerobes and in general quite sensitive to oxygen) within anaerobic sludge granules exposed to oxygen remained active at relatively high levels

for stable periods of time. Shen & Guiot (1996) have observed similar long-term effects on methanogenic/acetogenic granular sludge. In both studies, this tolerance to oxygen was mainly due to the activity of facultative anaerobes in the granule, which consumed oxygen at the surface and depleted it form inside, creating thereby extensive anaerobic zones. Such anaerobic zones within particles are not restricted to these few examples but is a rather widespread phenomenon in nature as it has been clearly demonstrated from measurements of steep oxygen gradients in gel beads (Figure 1), natural microbial aggregates (DeBeer et a, 1990,etc.), sediments (Daalsgaard *et al.*, 1987) and soil particles (Tiedje *et al*, 1984).

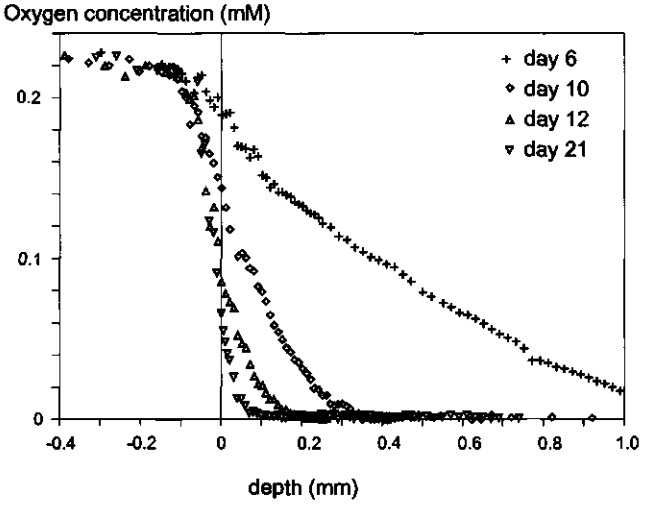


Figure 1 - Evolution of the oxygen concentration profiles along time in a support with immobilized nitrifiers. Adapted from Wijffels *et al.* (1995).

These few examples (see a more exhaustive description in Field *et al.*, 1995), clearly suggest that anaerobic and aerobic biodegradative capacities can be utilized synchronously in a sole environmental compartment. Since anaerobic microniches easily establish in aerated biofilms, one could devise ways of engineering biocatalyst particles so that multiple-step, complementary biotransformation and biodegradation processes would take place within a single carrier. Consequently, and quoting Field *et al.* (1995), "the best of both the anaerobic and aerobic worlds could be obtained in a single, compact bioreactor". The question of how to actually accomplish this will be, in what follows, illustrated for a compact system integrating nitrification and denitrification. Then, the opportunities for integrating other oxidative and reductive biodegrative processes will be addressed.

**Magic beads: an advanced engineering concept for process integration**

Based on the observation that such anaerobic niches formed within supports with immobilized nitrifiers, Tramper (1984) was the first to propose coupling nitrification and denitrification within double-layered beads. In such beads, the nitrifying microorganisms (aerobes) immobilized in an outer layer would oxidize ammonium into nitrite that would then diffuse inwards where immobilized denitrifiers (facultative heterotrophs) would reduce this nitrite into the harmful gaseous nitrogen (Figure 2). The product of the first microorganism is the substrate for the second one, and thus this two-stage bioconversion process is conducted as if single staged. The biocatalyst particle is used optimally because both the external layers and core are active. The beads are placed in a common airlift reactor through which the waste streams can flow at almost any rate, without the need of recirculation to or from any anoxic compartment or reactor.

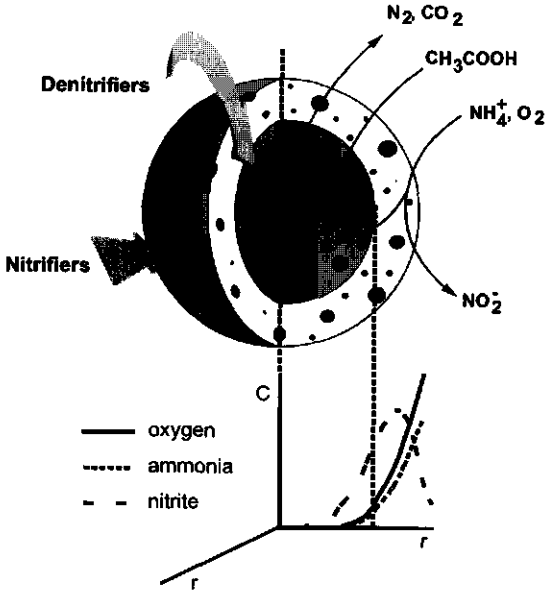


Figure 2 - The Magic-Bead Concept. Outer layer: nitrification; core: denitrification.

This process has been tested at bench scale with *Nitrosomonas europaea* and *Pseudomonas* spp. co-immobilized in κ-carrageenan. Martins dos Santos *et al.* (1996a, 1996b) have demonstrated that coupled autotrophic nitrification and heterotrophic denitrification could be indeed achieved at quite high rates in 3L air-lift loop reactors for a wide range of conditions. This reactor system performed well both under steady state

and dynamic conditions. Its behavior under both conditions was appropriately described by a dynamic model that predicted biomass and solute bulk concentrations, substrate consumption rates, product formation rates and both biomass and solute concentration profiles across the beads as a function of time (Martins dos Santos *et al.*, 1996). More detailed studies with specific microelectrodes for $O_2$, $NH_4^+$ and $NO_2^-$ showed that oxygen was indeed depleted after the first 100-300 μm from the surface, and that denitrification took place mainly beyond that distance (toward the centre) with no nitrate production.

Conceptually similar systems, in which nitrifiers and denitrifiers were co-immobilized as a mixed culture in single beads, had been also attempted with a relative success (Kokufuta *et al*, 1987, 1988, Tartakovski *et al*, 1996, Kotlar *et al*, 1996). However, despite being technically much simpler, the main problem of this approach (at least with this particular combination of microoganisms), is that denitrifiers, which are facultative anaerobes, tend to overgrow nitrifiers if co-immobilized as a mixed culture because they compete more successfully for oxygen and grow faster (Martins dos Santos *et al*, 1996, Kurosawa, 1990). Hence, physical separation between both populations has to be forced, reason for which double-layered beads were devised. In either case, it is noteworthy to emphasize that a potential disadvantage (development of anaerobic zones as a result of a rate limitation in aerobic bioconversion kinetics) has been successfully tackled and converted into a probably much greater advantage (integration of apparently incompatible processes within a "tiny" bioreactor).

***Advantages of magic-beads as compared to sequenced aerobic–anoxic reactors***

Besides the obvious benefits of achieving a complex, multistep process within a single, compact bioreactor, an important advancement is that the nitrifying populations can be selectively immobilized and hence nitrite can enter directly into the denitrification route. In this way, the intermediate steps involving nitrate are avoided with considerable savings in energy and resources. Indeed, in conventional wastewater treatment configurations, ammonium in the aerobic tank is normally oxidized into nitrite, which is subsequently converted into nitrate. In the subsequent anoxic tank, nitrate is again converted into nitrite (at expenses of organic matter) and this nitrite is finally reduced to nitrogen. In the "magic-bead" system, these two intermediate reactions are simply skipped. Recently, such concept has been further improved by co-immobilizing autotrophic nitrifiers (as above) and an autotophic ammonium oxidizing sludge that converts ammonium and nitrite directly to $N_2$ (Anammox, Van der Graaf, 1996, Strous, 1999). By using this combination, a fully autotrophic, compact system for complete

nitrogen removal could be set (Martins dos Santos *et al*, 2001c,d). Besides the enormous savings on both oxygen (50% as compared to conventional nitrogen removal) and organic carbon (100%), a tremendous advancement is that sludge production (and hence biomass surplus disposal) is strongly minimized due the very low yields and growth rates of the intervening populations. A third, and not less important, benefit is that pH control is greatly facilitated because part of the acidity produced by nitrification is rapidly compensated by the alkalinity resulting from denitrification. These effects (that make the system intrinsically much more stable and robust) have been quantitatively predicted by Sheintunch and co-workers (Tartakovski *et al.*, 1996) and Martins dos Santos *et al.* (2001b) and were also experimentally verified using specific pH-microelectrodes together with the above mentioned nitrite, ammonium and oxygen microelectrodes (Martins dos Santos *et al*, 2001a,b).

Finally, a relevant additional advantage is that the global nitrogen removal rate in co-immobilized systems may be enhanced because the nitrite produced in nitrification is immediately removed by denitrification. Hence, possible inhibition effects by nitrite (or $HNO_2$ for lower pH) are minimized, which is especially important for heavily loaded streams (Martins dos Santos, 2001b). Similarly, it has been suggested that the facilitated pH control within the biotatalyst particles themselves (flatter pH gradients) would allow faster ammonium oxidation as compared to pure nitrifying cultures (Tartakovski, 1996). This may be indeed so for poorly buffered streams, as experimentally demonstrated by Martins dos Santos and colleagues (2001b). This effect of coupling reaction with intermediary removal within the carrier (analogously to integrated reaction – separation for whole processes) may be actually one of the greatest advantages of combining multistep processes in general within a single biocatalytic particle.

**Coupling "Red - Ox" processes**

***Classics***

The "magic-bead" concept for compact nitrogen removal just addressed illustrates a strategy that could be, in principle, extended to many other biodegradation and biotransformation processes that frequently require sequenced reduction and oxidation.

In some cases, this opportunity has been already explored and some combinations have been assessed. For instance, in 1986 Tanaka and co-workers described the direct production of ethanol from starch by the synergistic action of co-immobilized *Aspergillus awamori* and *Zymomonas mobilis*. Similarly, in 1987 Kokufuta *et al.*

reported on batch-wise integrated nitrogen removal by *Nitrosomonas europaea* and *Paracoccus denitrificans* co-immobilized as a mixed culture.

The first application to xenobiotic degradation, however, was that presented by Beunink & Rhem (1988), in which a DDT dechlorinating bacterium (*Enterobacter cloacae*) was co-immobilized into calcium alginate beads together with the *Alcaligenes* spp. strain Iso DPM4 (an obligate aerobe able to co-metabolize DDM with dimethylmethane as a primary substrate). Such a co-immobilized co-culture was placed into an aerated fermentor and was able to simultaneously dechlorinate DDT and mineralize DDM. That dechlorination was possible under the prevailing aerobic was attributed to the anaerobic microniches in the centre of the gel beads. In a follow-up study, a similar co-culture with *E. Cloacae* and another *Alcaligenes* sp. strain (TK-2) was constructed for the synergistic biodegradation of 4-chloro-2-nitrophenol (4C2NP) (Beunink & Rhem, 1990). The results of the study indicated that *E .cloacae* present in the bead core successfully reduced 4C2NP to 4C2AP (4-chloro-amino-phenol) and 4C2AAP (4-chloro-acetaminophenol), which were then aerobically mineralized by the *Alcaligenes* that had accumulated preferentially at the outer layer of the beads.

Analogously, the restriction of oxygen by diffusion within immobilization supports was exploited to produce coupled reductive and oxidative degradation of 2,4,6-trichlorophenol (2,4,6TCP) (Gardin & Pauss, 1994). As above, 2,4,6TCP was first anaerobically converted into 4-chloro-phenol, which was then mineralized by an aerobic population at the periphery of the bead.

Another classical example is that regarding the complete elimination of 2,3,6-triclhorobenzoic acid in a hybrid (suspended - immobilized-cell) reactor (Gerritse & Gottschal, 1992). An anaerobic enrichment able to dechlorinate 2,3,6-triclhorobenzoic acid (2,3,6TCBz) to 2,5-dichlorobenzoic acid (2,5DCBz) was immobilized on a vermiculite support and placed in a continuous reactor to which a 2,5DCBz mineralizing aerobe, *Pseudomonas aeuroginosa*, had been added. The aerated co-culture was able to completely mineralize 2,3,6TCBz with 95% recovery of organochlorine as $Cl^-$. Here, the aerobic microorganism was mostly suspended but its oxygen-consuming activity was nevertheless strong enough so to ensure that the bulk oxygen concentration remained within 0.013 to 0.03 $mg.L^{-1}$, apparently low enough to allow dechlorinating activity inside the vermiculite supports.

It is noteworthy to stress that such reductive-oxidative systems may be, in fact, potentially even more effective than that described for nitrogen removal by "magic beads". Indeed, in processes where the first degradation step is anaerobic or anoxic, the

pollutant diffuses first towards to the bead core. There, it is converted into their intermediates that subsequently diffuse outwards and are mineralized as they reach the aerobic outer zones. Therefore, full mineralization within a single bead is expected to occur. On the contrary, in systems such as that of integrated nitrogen removal described above, the pollutant is first aerobically converted to their intermediates in the outer layers, which then diffuse both inwards and outwards (see profiles in Figure 2). Hence, such a full decontamination process should necessarily account for this "extra" fraction of (initially) unconverted intermediates.

***Xenobiotic Red-Ox systems***

Similarly to the examples above indicated, there are many other compounds that may be effectively eliminated by this approach. As referred above, many complex compounds are recalcitrant to oxygenolytic attack but are nevertheless amenable to anaerobic reduction. Their products on the other hand are resistant to further reduction but are readily oxidized by aerobic microorganisms. In what follows, the possibilities of coupling oxidative and reductive conditions within a single carrier will be discussed for a series of xenobiotic families. Most of the compounds below have been already reported as being potentially biodegradable by specific microbial communities.

*PCB's*

Polychorinated biphenyls, likewise most halogenated organic compounds are xenobiotics and therefore quite recalcitrant to mineralization. Such compounds (e.g. Arochlor series) are often used as pesticides and consist of complex mixtures of different PCB's (there are 209 possible isomers of which about 100 exist in commercial formulations. All are synthetic.). Besides bioavailability problems, the main reason for recalcitrance is that natural evolution has not yet been fast enough so to provide microorganisms with the right enzymatic machinery for tackling effectively such compounds or mixtures of them. There are, however, microorganisms able to dechlorinate reductively highly chlorinated PCB's yielding thereby less chlorinated products. These products are difficult to degrade further anaerobically but are readily oxidized by aerobic bacteria. A general trend is that the more halogenated a given compound is then the more recalcitrant is it to aerobic or anoxic biodegradation but, inversely, the more amenable is to reductive dehalogenation (Field *et al*, 1995, Zitomer & Speece, 1993,Chaudhry & Chapalamadugu, 1991). Several aerobic and anaerobic microorganisms, naturally isolated and genetically modified, have been extensively studied and described in regard to these processes (Quensen *et al*, 1988, Havel &

Reinecke, 1991, Chaudhry & Chapalamadugu, 1991, Commandeur & Parsons, 1993). As yet, however, and despite abundant information, only few systems had been reported to make use of these combined processes for removing PCB's from waters and soils. In even fewer cases, two-stage systems have been devised (Anid *et al*, 1991, Bédard et. al, 1987). It is however straightforward to think that coupling reductive dehalogenation to aerobic oxidation may be possible and worthwhile in many instances.

Hypothetically, a suitable artificial consortium could be constructed in which reductive dechlorinating microorganisms are co-immobilized with bacteria able to mineralize the less chlorinated products of the dechlorination step. An example would be the dechlorination of mixtures of tetra- and pentachlorobiphenyl to dichlorobiphenyls within the core of a biocatalytic particle, which would be then aerobically mineralized in its the outer layers. Such specialized biocatalysts could be placed in aboveground bioreactors and used for the on-site treatment of contaminated groundwater.

Other possibility would be placing in soils strategically designed sequential biological screens containing the relevant populations for complete PCB mineralization. Aeration would be provided to screens for the degradation of the lower chlorinated products of anaerobic dechlorination. Such screens would be placed strategically so to intercept contaminated groundwater. A more detailed discussion of systems for *in-* and on-site treatment of contaminated waste- and groundwater is discussed later in this work.

*Other halogenated compounds*

Much of what was said above for PCB's and for their full biodegradation in compact systems applies entirely to many other halogenated contaminants. Such compounds include both aromatic (single and polycyclic) and aliphatic hydrocarbons. All these halogenated compounds (including PCB's) have been widely used, or are themselves undesirable by-products, in the manufacturing of chemicals, industrial solvents, pesticides, insecticides, herbicides, etc.

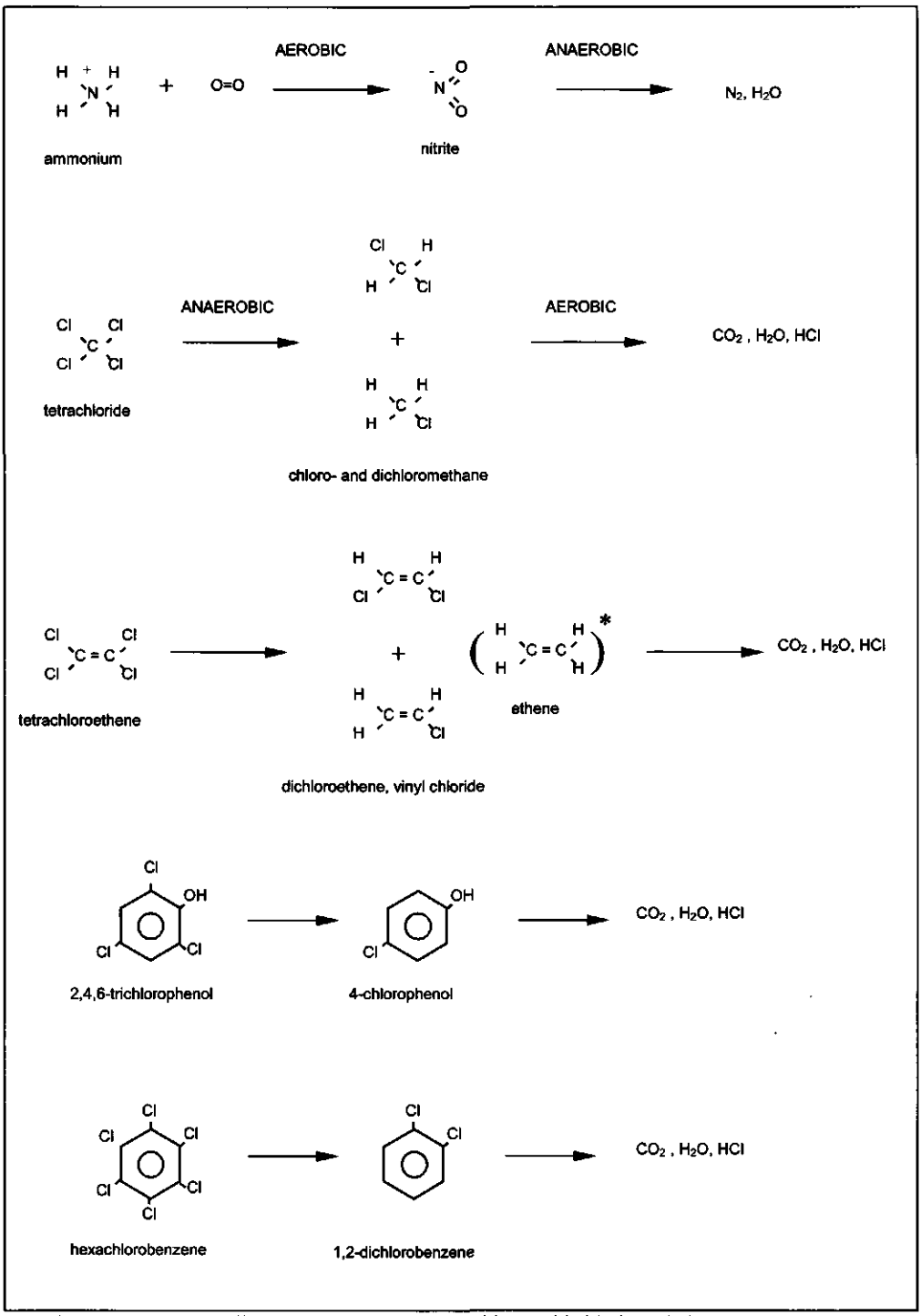


Figure 3 – Examples illustrating sequenced anaerobic-aerobic biodegradation processes.

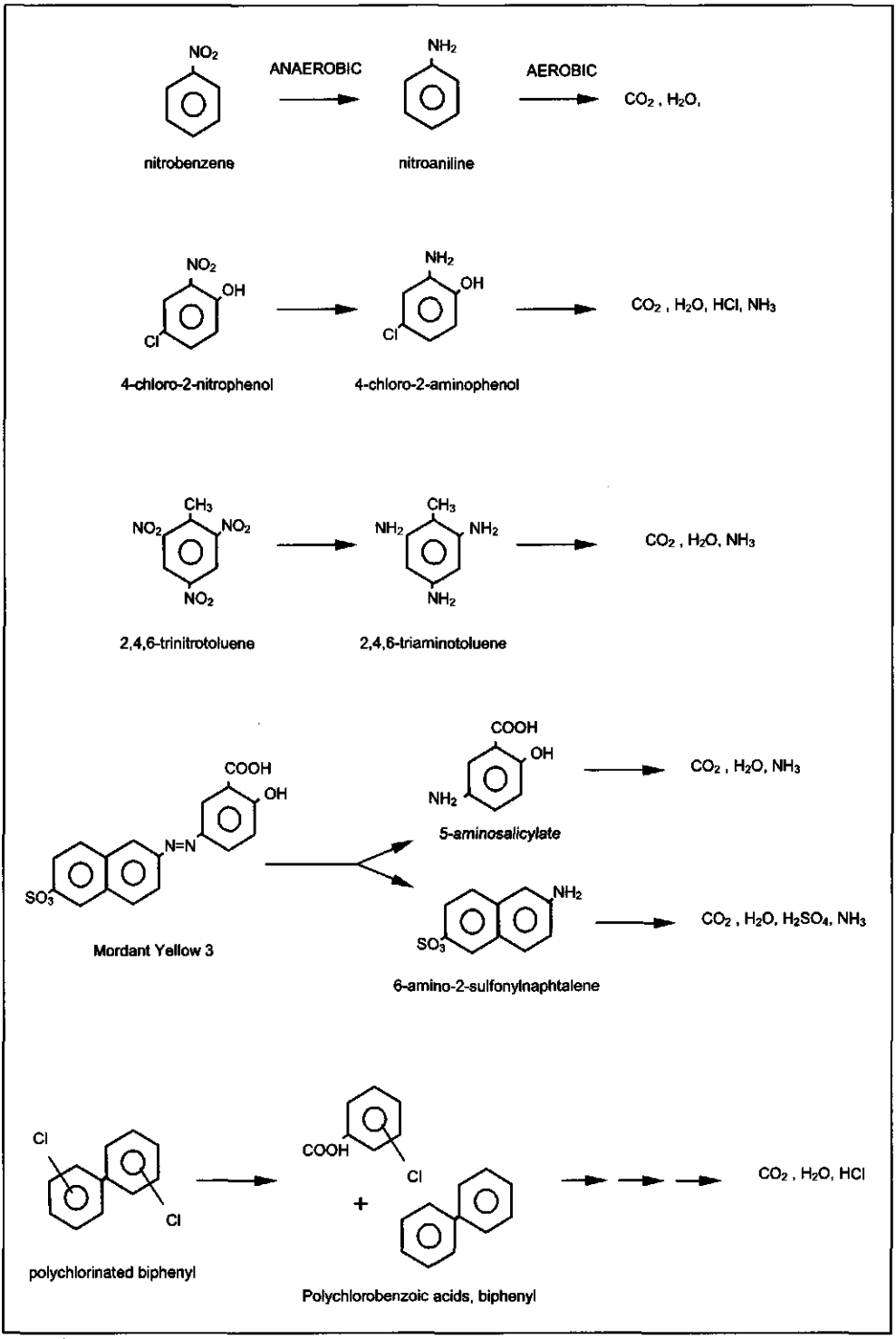


Figure 3 – Examples illustrating sequenced anaerobic-aerobic biodegradation processes (continued).

*Aromatics*

Examples of recalcitrant halogenated aromatics include polychlorinated phenols, polychlorinated benzoates, fluor and bromobenzoates, hexachlorobenzene, methoxychlor, among many others (see Zitomer & Speece, 1993, Field *et al.*, 1995 and Chaudhry & Chapalamadugu, 1991, for more exhaustive descriptions). In this regard, some anaerobic-aerobic sequences have been already described in literature. For instance, Hakulinen & Salkinoja-Salonen (1982) reported effective treatment of bleachery effluents containing mixtures of toxic chlorophenols by an anaerobic fluidised bed reactor coupled to an aerobic trickling filter. Also compounds such as methoxychlor (1,1–bis(p-methoxypehnyl)-2,2,2-trichloroethane) or hexachlorobenzene have been shown to be eliminated with relative success using anaerobic-aerobic sequences (Fogel *et al*, 1982 and Fathepure & Vogel, 1991, respectively). Another known example is that of the degradation of 2,4,6-trichlorophenol (2,4,6TCP) and other chlorophenolic compounds, which have been already successfully tested both in anaerobic-aerobic sequences (Fahmi et. al, 1994, Kafkekwitz et. al. 1992, Kettunen & Rintala, 1995) and in two-stage anaerobic-aerobic reactors (Armenante *et al.*, 1999). The first reported attempt to couple these sequences within a single biocatalyst bead was that presented by Gardin & Pauss (1994), in which 2,4,6TCP is reduced to 4-chlorophenol and subsequently mineralized aerobically (see above). Similarly, Wilson *et al.* (1997) have reported on anaerobic/aerobic pentachlorophenol degradation using granular active carbon in fluidized bed bioreactors.

For many other halogenated aromatics (e.g. polychlorinated dibenzodioxines), however, still very little is known regarding their biodegradative possibilities but it is likely that the general trend would be very similar. Even not knowing yet what microorganisms or consortia may play a role in each step of their biodegradation, it could be expected that, whenever these are found, either similar anaerobic-aerobic sequences or magic-bead-like processes could be applied.

*Aliphatics*

Likewise the just mentioned examples of aromatic halogenated compounds, there are many halogenated aliphatics that could be, hypothetically, treated in compact systems such as those proposed here. These are mainly polyhalogenated alkenes, alkanes and alkanoic acids. Again, these groups of compounds are in general readily dechlorinated anaerobically whereas their less chlorinated products are more amenable to degradation under aerobiosis. In many cases, (partial) biodegradation occurs as a result of

cometabolic processes involving the breakdown of simple organics like weak organic acids, methane, propane, toluene, phenol, etc.

Biodegradations of tetra-, tri- and dichloromethane, for instance, have been subjected to rather intensive investigations because they are widespread toxics. Tetrachloromethane (carbon tetrachloride, CT), although resistant to biodegradation, it is in general readily dechlorinated to chloroform or/and dichloromethane under anaerobic conditions (de Best, 1998 *et al*, Criddle & McCarty, 1991). Straightforwardly, one would thus expect that subsequent aerobic treatment by means of appropriate microbial populations would lead to its full mineralization. Indeed, chloroform and dichloromethane have been shown to be amenable to aerobic co-metabolic degradation (Semprini, 1997). Such sequenced anaerobic-aerobic treatment may be crucial not only for CT itself (which has sometimes been reported to undergo almost complete degradation, though at low rates (van Eekert *et al*, 1998)), but especially for mixtures of chlorinated alkanes and alkenes because of the increased complexity for the degrading populations. One such sequenced approach has been indeed reported for the treatment of mixtures of chlorinated aliphatics in a two-stage bioreactor system (Long & Stensel, 1990). Yet, very few other studies regarding such sequences have been done. Also, no study regarding the integration of these sequences within a single particle has been reported yet.

Likewise their alkane counterparts, halogenated alkenes have been subjected to rather intensive research. Particular attention has been paid to tetra- and trichloroethylene (PCE and TCE, respectively) because of both its toxicity and abundance. Both PCE and TCE are more effectively handled under anaerobic than aerobic conditions (mostly as a result of fortuitous, co-metabolic reactions, but fermentative metabolism has been reported as well). Again, the opposite is true for their less chlorinated products. In the view of the development of integrated treatment processes, several groups have been attempting (apparently with success) to couple reductive dechlorination of PCE (or TCE) to DCE (dichloroethylene) and/or VC (chloride) sequentially to aerobic mineralization of DCE and VC (Gerritse *et al*, 1995, Arvin *et al*, 1995). In one system (Arvin *et al*, 1995), a biofilm reactor with a new PCE-utilizing anaerobe (*Dehalospirillum multivorans*) is placed in series with a biofilm reactor containing a microorganism growing on VC and degrading DCE co-metabolically (*Methylosinus trichosporium* OB3b, van Hylckama-Vlieg *et al*, 1996). In a similar configuration (Gerritse *et al.*, 1995), a dechlorinating, methanogenic enrichment in a biofilm reactor was placed in series with a biofilm reactor containing a methanotrophic enrichment able to metabolize the less chlorinted ethenes.

The first attempt to combine these processes within a single biocatalytic particle has been recently reported by Tartakovki *et al.* (1998), who used a consortium of co-immobilized methanogenic and methanotrophic bacteria for elemination of PCE.

*TNT*

Similarly to halogenated compounds, TNT, RDX and other nitrosubstituted aromatics tend to be rather recalcitrant to due to the high electron-withdrawing character of the nitrogroups on the aromatic ring. TNT is a major toxic in many sites throughout Europe and USA as a result of the intensive army industrial activities. Its elimination has proved quite difficult and full mineralization (or at least as high as 20 %) has never been reported. Yet, in many instances it has been shown that TNT may be either incorporated in biomass and or is transformed anaerobically into aminotoluenes, of which the main component is the triaminotoluene (TAT, Knackmuss, 1996, Rieger & Knackmuss, 1995). TAT itself is rather unstable and can be degraded further into products that are in turn mineralized aerobically (Funk *et al*, 1993). Hence, a two-stage TNT elimination has been proposed. However, due to its instability, TAT also dimerizes and polymerizes in the presence of oxygen to give azoxy dimers, which are quite toxic themselves (even more then TNT). Therefore, a truly two-stage anaerobic/aerobic treatment is deemed to failure. In that regard, the only improvement done so far was that reported by Daun *et al.* (1995), who proposed an anaerobic/aerobic system for soil treatment. In this system, indigenous populations first convert anaerobically TNT into TAT (providing as well that a co-substrate is added). Subsequently, by providing aerobiosis, TAT binds irreversibly to clay or the humic acid fractions of soil or carrier particles.

Hypothetically, however, one would expect that the application of a magic-bead-like process to TNT elimination could lead to true mineralization. Providing that the pertinent microbial populations are present, one could hypothesize that TNT would be also transformed in to TAT and other aminotoluenes within the bead core. However, unlike the Daun's process, it would be expected that the TAT and their products would not accumulate significantly because the aerobic microorganisms present in its vicinity would remove them as soon as they form. Therefore, little or no polymerization would be expected. In this way, such a "magic-bead"-like system would thus not only improve an existing configuration but, more importantly, it would make possible a process (significant TNT mineralization) not feasible otherwise.

*Nitrosubstituted aromatics*

Many nitrosubstituted aromatics, due to its electron-withdrawing group are also resistant to full mineralization either by aerobic or anaerobic microorganisms alone. In that regard, a few sequenced anaerobic-aerobic degradation processes has been already reported for several of such compounds. For instance, such combined processes have been suggested for the elimination of nitrobenzene (Dickel *et al.*, 1993, Peres *et al*, 1998). In those processes, nitrobenzene is anaerobically converted into aniline, which is in turn metabolized aerobically. According to a "magic-bead" scheme, this process could be, hypothetically, accomplished within a single biocatalytic support with appropriate co-immobilized microorganisms.

*Azo-dyes*

Azo dyes, which comprise the most important family of compounds used in textile dying, are generally quite recalcitrant to aerobic degradation due to their characteristic azo bond (N=N). Yet, this highly electrophilic bond can be cleaved anaerobically by several bacteria. The cleavage products (aromatic amines and derivatives thereof), however, generally resist further anaerobic degradation because the electron-donating amino groups now hinder a nucleophilic attack. Luckily, these aromatic derivatives (even those containing sulfonate groups) are readily mineralized by some aerobes. Taking advantage of these characteristics, several schemes of sequenced anaerobic-aerobic degradation of azo dyes have been tested. For instance, Haug *et al.* (1990) have found that a 6-aminonaphtalene-2-sulfonic acid (6A2NS) degrading consortium was also able to reduce the sulfonated azo dye Mordant Yellow 3 (MY3) under anaerobic conditions. The products of this reduction were 6A2NS and 5-aminosalicylate, which easily undergone mineralization after aeration. Hence, by applying alternate anaerobic and aerobic conditions to the suspended-cell reactor (batch), MY3 could be fully mineralized. Later, Glasser *et al* (1992) improved this sequence by setting up an anaerobic expanded-bed reactor in series with an airlift loop reactor. Further compaction of this system has been recently described by Kudlich *et al.* (1996), who used *Sphingomonas* sp. BN6 and a 5-aminosalycilate degrading bacterium (5AS1) co-immobilized within alginate beads for attaining complete mineralization of MY3. Within the anaerobic bead core, *Sphingomonas* sp. BN6 reductively cleaves the azo yielding 6A2NS and 5-aminosalicylate. 6A2NS is then oxidized in the outer layers to 5-aminosalicylate, which in turn is mineralized by 5AS1.

The analysis just made for MY3 degradation may set the basis for a rational approach towards the development of a compact, integrated system for the treatment of azo dyes

and, more importantly, for mixtures of them (which is actually the most common situation). Examples of other possible candidates would be Disperse Red 7, Azobenzene, Mordant Orange 1, Acid Yellow 25, and so forth.

*Others*

The reasoning behind the "magic-bead" concept is not only applicable to the integration of oxidative and reductive systems but also to the design of microbial consortia that may perform any given multi-step transformation and not necessarily under different environments. As it is further described in the subsection "microbial consortia", such constructions (and the underlying models describing them) are useful for the assemblage of metabolic pathways for the degradation of complex mixtures of toxic compounds. Hence, specialized consortia could be made for the degradation of mixtures of substituted aromatics (nitrobenzoates, nitrophenols, etc), mixtures of sulfides, mixtures phenols and cyanides, etc.

**Mechanistic models as tools for the design of integrated oxidative/reductive processes**

Mechanistic models are very valuable tools for both the understanding and for the design, control and scale-up of complex systems, and are as well essential for the prediction of the responses of a given system to changes in environmental and operating conditions. Also, they also give quite much insight into the mechanisms underlying the processes under consideration. This insight is extraordinarily valuable in the sense that it allows us to understand into great detail the basic phenomena regulating the processes involved, and gives us a rather powerful tool to control, modify or extend them to other systems in which these or similar phenomena play a role. Finally, they provide us with a solid knowledge framework in which information regarding complex interactions can be organised and systematised.

These arguments regarding modelling of bioprocesses in general are particularly relevant when applied to the (intrinsically very complex) coupled oxidative - reductive systems above described. Indeed, understanding and identifying the most relevant interactions among the different microbial types, their metabolisms and activities, as well as determining the influence of the many different physical, chemical and mechanical factors on their behaviour and on the system's performance as a whole, requires a great deal systematisation and fundamental knowledge. Hence, a few different models for describing various aspects of such synchronous processes have been developed by a series of different groups.

***Magic beads as a prototype for modeling Red – Ox processes***

In attempt to describe the complex interdependencies underlying coupled nitrification and denitrification within magic beads a mechanistic model was developed accordingly (Martins dos Santos et. al, 1996). This dynamic model accounted for diffusion of components, substrate utilization and growth, all occurring simultaneously in the beads. For modeling purposes, different substrates could be limiting within each conceptual shell of the beads. Both internal and external mass transfer resistance was accounted for as well. The model predicted biomass and solute bulk concentrations, substrate consumption rates, product formation rates, and biomass and solute concentration profiles within the beads as function of time. Intrinsic kinetic parameters of the microorganisms, internal and external mass transfer coefficients, initial conditions, bead concentration and particle diameters were the input parameters. Fluctuations in substrate load, dilution rates or mass transfer parameters could be accommodated as well. To be of practical use, any mathematical model, irrespectively of its conceptual importance or predictive capacity, needs necessarily to be validated experimentally. Only then can its outcome or predictions be seriously taken into account for the description of a system. Accordingly, this model was validated both at reactor and biocatalyst level. At reactor level, validation was done by measuring the macroscopic consumption and production rates of co-immobilized cells as a function of time, both under steady state and dynamic conditions (Martins dos Santos *et al*, 1996b). The model was validated at particle level by measuring substrate and product profiles across the gel beads using polarographic ($O_2$) and ion-selective ($NO_2^-$ and $NH_4^+$) microelectrodes (Martins dos Santos *et al*, 2001a). At both levels, model predictions described well experimental results.

Figure 4 illustrates well the scope of such modeling and validation work for both nitrifying and coupled nitrifying/denitrifying conditions. Beyond the first few hundreds μm from the surface of the beads there is practically no oxygen left. The nitrifying activity occurs mainly in this region and the rest of the bead remains unused in the absence of an organic C-source, as shown by the flat profiles of nitrite and ammonia (4A). If an organic C-source is supplied to the system, denitrification takes place and a nitrite gradient throughout the bead will develop as well (Fig. 4B). In the simulation presented here, oxygen is the limiting substrate (note the different scales) for nitrification and it will thus limit the overall nitrogen removal rate since acetate is supplied in excess. Clearly, the predicted and measured profiles agree well with each other, which evidences that the model is indeed appropriate to describe the behavior of the proposed process.

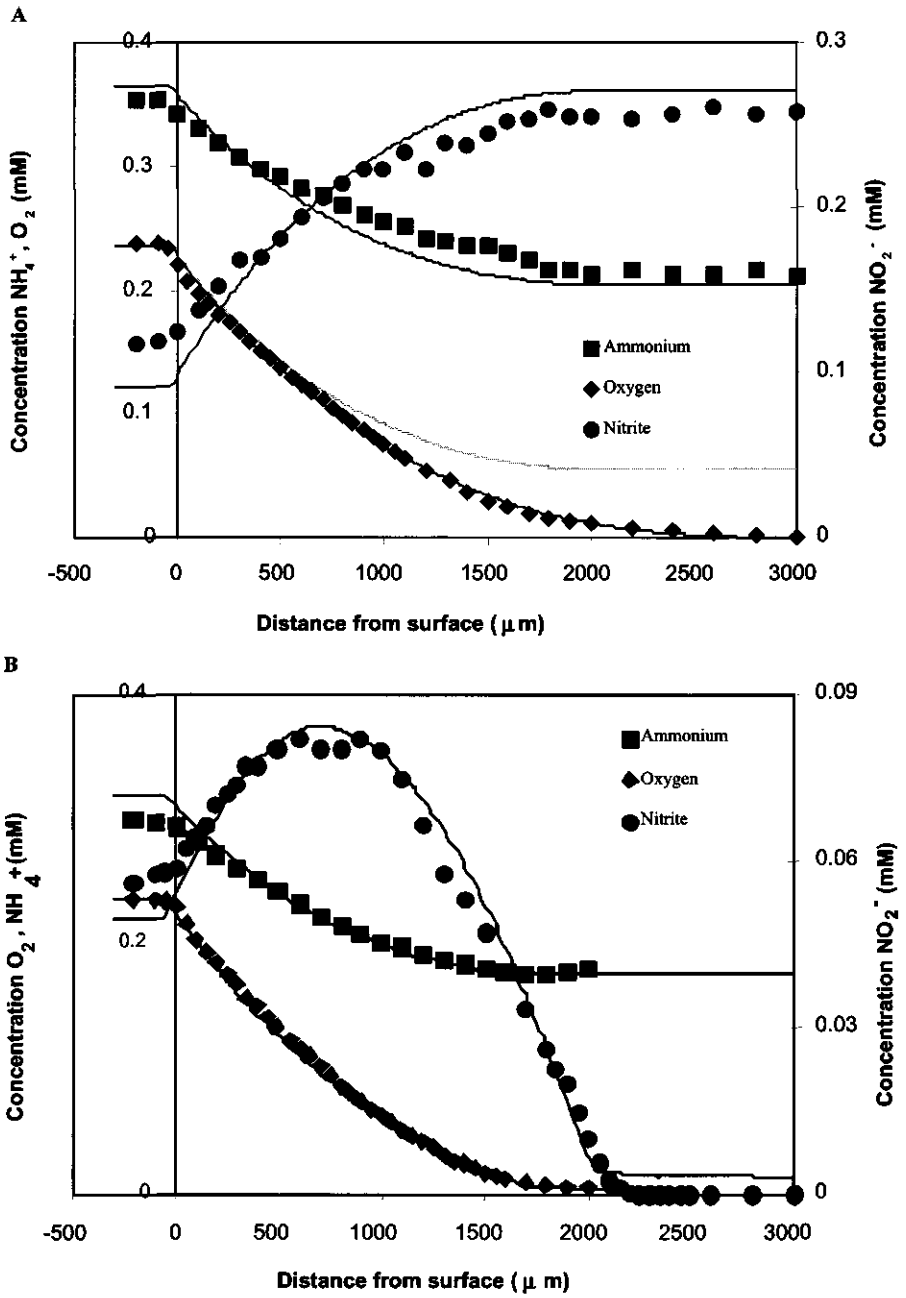


Figure 4 - Typical set of measured (symbols) and predicted (lines) substrate and product profiles for beads under nitrifying only (A) and nitrifying plus denitrifying (B) conditions (i.e., in the absence and the presence of an organic C-source, respectively).

Later, this model was extended so to account for the pH effects as a result of nitrification, denitrification and synchronous nitrification/denitrification under several buffering conditions. Also under these conditions were substrate, product and pH profiles well predicted by the model (Martins dos Santos *et al*, 2001a,b). Both these predictions and experimental results also compared well predictions earlier done by Tartakovsky *et al.* (1996), who developed a similar model for coupling nitrification and denitrification with co-immobilized mixed culture of nitrifying and denittrrifying bacteria.

***Applicability of models of general character***

The model just described is founded on general process equations and on the intrinsic kinetics of the intervening cells or microbial groups. Therefore, this or similarly general models (such as those reported by Meyerhoff *et al*, 1998, Tartakovsky *et al*, 1998, Peng & Bly, 1998) would be, at least in theory, easily adapted so to describe other systems providing that a few relevant parameters and some key interrelationships are known. Indeed, such an extension has been successfully done for describing the activity of a co-immobilized amyloglucosidase and *Zymomonas mobilis* (Hellendorn *et al.*, 1999) and that of co-immobilized *Aspergillus niger* and *Zymomonas mobilis* (Hellendorn, 1999, Meyerhoff *et al*, 1998) for the production of ethanol from starch-related compounds. Similarly, the dynamic behavior of a fully autotrophic nitrogen removal system in which Anammox and nitrifying sludge were co-immobilized as a mixed culture could be rather satisfactorily described by such a model (Martins dos Santos *et al.*, 2001e,f). Interestingly, in this study not only nitrogen removal as such was adequately handled but also the interactions (both synergy and competition for substrates) among the three main microbial groups were correctly handled. This outcome suggests thus that the model may be also adequate for describing the behavior co-immobilized microorganisms in general and not specifically for oxidative-reductive processes. Preliminary work has shown that this model could indeed predict the population shifts and syntrophic relations between sulfate reducing bacteria and methanogenic producers within anaerobic granular sludge (Martins dos Santos VAP, Lens P, Wijffels RH, unpublished results). Similarly, the syntrophic interdependencies among the members of an anaerobic consortium degrading haloharomatics could be reasonably well described (Martins dos Santos VAP, Maarsen K, Stams AJ, unpublished results). Finally, this same model could be successfully adapted so to describe the transient activity of co-immobilized bacteria mineralizing a mixture of *meta*- and *para*-nitrobenzoate (Goodall *et al*, 1998a,b).

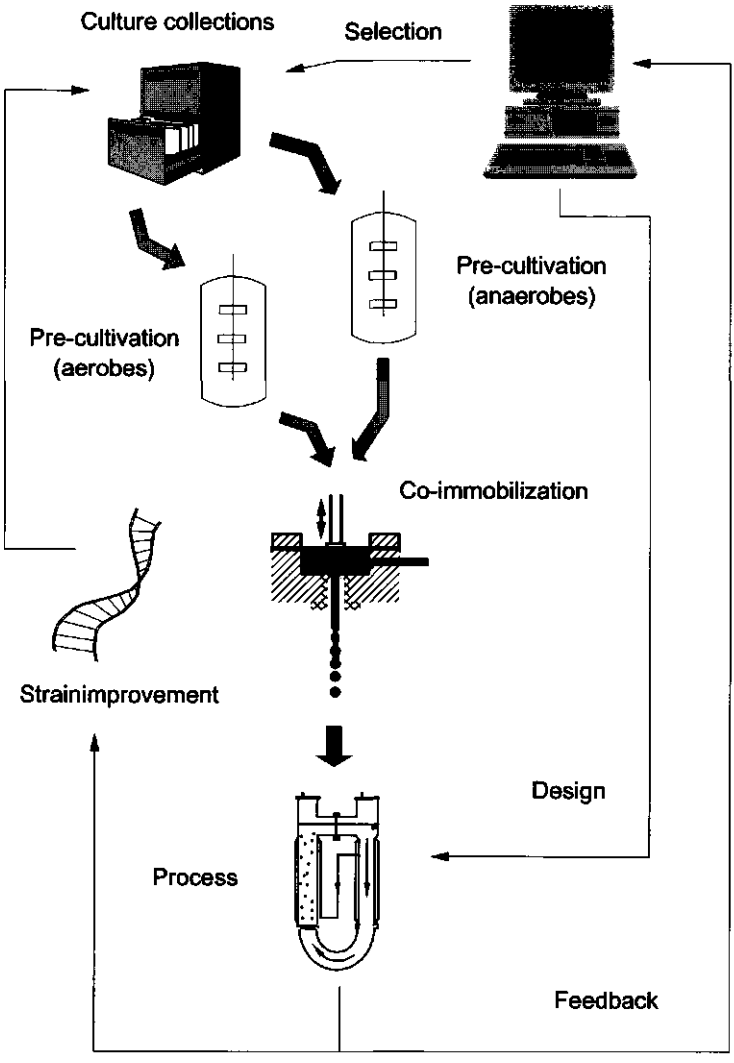


Figure 5 – General strategy proposed for the design of compact oxidative-reductive processes.

This series of examples clearly show that mechanistic models may indeed have a general applicability regarding coupled oxidative reductive processes. Besides the insight they give, they can be also essential in evaluating, beforehand, whether a given hypothetical process is worth investigating in practice and whether it may present potential (technical) advantages over existing sequenced processes. Thereby, much

experimental and economic efforts may be saved and investigation toward compact systems can be more rationally designed.

***Two-stage versus single reactor system***

Once the model is relatively well established, simulations for the systems proposed below (next subsection) should be performed and compared, within a wide range of conditions, to simulations of the same systems in two-stage reactor configurations. This extensive set of simulative work would provide a rather sound basis for the choice and experimental design of the candidate systems.

**Genetic engineering as a tool for the design of effective biocatalysts**

Despite the improvements and benefits possibly arising from the integration of reductive and oxidative biodegradation processes, the microorganisms responsible for various of such processes still have to cope with the intrinsic recalcitrant character of many xenobiotics. With increasing complexity of a xenobiotic or mixtures of them, we cannot expect to find complete (co-)metabolic pathways in a single organism or even in a simple set of them. In other words, even a careful construction of a defined consortium within a given biocatalytic particle and operation under the most appropriate environmental conditions may be insufficient to fully overcome the intrinsic recalcitrance of given contaminants or mixtures thereof. As discussed above, the cause is often related to either inherent toxicity (e.g. polar organic solvents) or to molecular bottlenecks such as are incomplete pathways leading to the formation and accumulation of dead-end (toxic) metabolites, inappropriate regulation of catabolic pathways or simply poor intrinsic transformation rates.

***Directed evolution***

Where the exploitation of syntrophic interactions among different members of a given (either natural or designed) consortium is not possible or satisfactory, genetic modification of pertinent microorganisms may be essential. However, as already discussed above, leaving to nature the role of modifying and improving microorganisms so that bottlenecks are overcome may just take too long, particularly when the acquisition of multiple catabolic activities is necessary. An alternative to this is to direct and/or accelerate evolution by experimentally designing new pathways. Evolution of new or improved activities in the laboratory may be a valid alternative because the frequency and types of genetic events needed (mutations, alteration of gene expression,

gene dosage, gene transfer and so forth) can be carefully controlled under selective and appropriate conditions (Liu & Suflita, 1993, Timmis *et al.*, 1994).

In this regard, molecular biology offers very valuable tools for circumventing or at least improving many of the above mentioned bottlenecks. By employing a variety of recently introduced genetic and protein engineering techniques, new and/or more effective biodegradative abilities can be acquired by assembling pre-existing enzymes in novel combinations, by modifying their expression or substrate specificity, by enhancing its genetic stability (Timmis *et al*, 1994, Pieper & Reinecke, 2000, Arnold, 1996, Ensley, 1994, Suflita & Kiu, 1993, Harayama *et al.*, 1998). More specifically, such approaches have been used to: i) expand the substrate range of existing pathways through both the recruitment of isofunctional enzymes (horizontal expansion) and the grafting of additional enzyme activities (vertical expansion) (Lehrbach *et al*, 1984); ii) restructure existing pathways to avoid generation of deleterious metabolites (Ramos *et al*, 1987); iii) construct new metabolic activities by patchwork of assembly of genes from different microorganisms that encode desirable enzymatic conversions (Rojo *et al*, 1987, Reinecke, 1998); iv) construct new gene sequences by enhancing natural genetic exchanges between dissimilar microorganisms with imposed selection pressure (molecular breeding) (Kellog *et al.*, 1981).

***Hybrid pathways***

One of the most relevant applications of directed evolution to biodegradation is the construction of hybrid strains that harbor complementary enzyme activities. These enhanced activities may be complementary catabolic sequences *sensu-estrictu* or broadened substrate specificities (Ensley, 1994). Striking examples of this strategy are the construction of hybrid strains for the degradation of halogenated aromatics (Rojo *et al.*, 1987, Timmis & Pieper, 1999, Reineke, 1998), trinitrotoluene (Duque *et al*, 1993), chlorinated alkenes (Furukawa *et al.*, 1994) or halogenated alkanes (Bosma *et al.*, 1999). In all these studies, either the substrate range was successfully expanded or a "new" route (i.e. a route not yet found in nature) was developed on the basis o f existing catabolic segments.

It should be pointed out, however, that genetic manipulations by themselves are not likely, as yet, to lead to the combination of oxidative and reductive activities. Indeed, the metabolic pathways involved are often too different or thermodynamically unfavorably to allow a straightforward combination of activities. Genetic engineering may, nevertheless, greatly improve or extend each of the oxidative and reductive activities so that their combination is strongly facilitated. One example would be the expansion of

the reductive dehalogenation capacity of a given microorganisms (e.g. one that would be able to multi-dehalogenate a PCB or haloalkene instead of its first or second substituent only) in a sequential reductive-oxidative process for the mineralization of a multisubstituted halogenated pollutant. Furthermore, natural evolution is itself not standing still as demonstrated by the discovery, shortly after the Gulf war, of microorganisms able to tackle some of the recalcitrant oil products that resulted from large scale spills, or by the more recent finding of a bacterium that reductively dechlorinates tetrachloroethene to ethene (Maymó-Gatell *et al*, 1997). This shows that research efforts should not be directed only to the development of fancy genetic engineering techniques, but also to the rational design of strategies for the selection and cultivation of microorganisms of environmental interest.

**Implications for *in-situ* and on-site bioremediation**

We believe that the concept presented here for the integration of oxidative and reductive activities has certainly a great potential toward its application to the treatment of contaminated streams or to the bioremediation of contaminated sites. The practical implementation of this concept, when to be applied to any of the processes described above would certainly depend on many technical and economical issues. However, for the sake of illustration in Figure 6 we schematically show a series of possible generic applications.

The most straightforward application, as discussed above, would be the implementation at large scale of the fully autotrophic system upon which the whole concept was developed for the effective removal of nitrogen from wastewater. Besides this application, conceptually, a system consisting of a compact reactor(s) filled with biocatalytic particles in which the relevant microorganisms are co-immobilized, could be used for the end-of-pipe treatment of industrial streams heavily loaded with waste contaminants or toxic side-products (upper part of Figure 6). In another context, such a system could be as well applied to on-site bioremediation of, for instance, contaminated aquifers or groundwater (middle). Indeed, above-ground reactors coupled to a pump-and-treat process can be rather appropriate for bioremediation (Alexander, 1994). In this process, contaminated water is extracted, treated above ground and subsequently pumped again into its shed. These reactors may be mobile units taken to field sites and used to treat groundwater pumped out of a contaminated aquifer or wash water used to remove chemicals from contaminated soils.

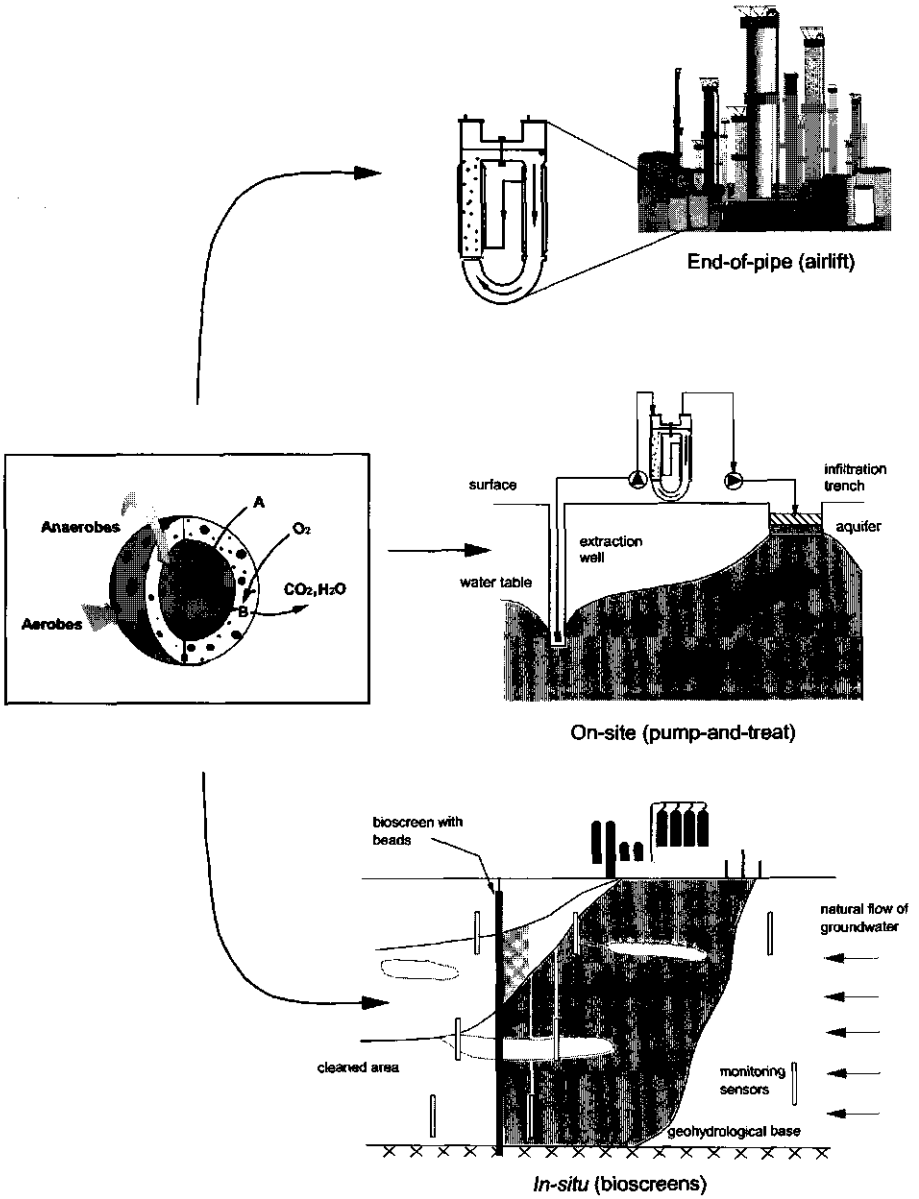


Figure 6 – Application of the "magic-bead" concept for end-of-pipe treatment of contaminated streams (A) and in situ (B) and on-site (C) bioremediation of polluted sites.

A further application would be the use of such biocatalyst particles for in-situ bioremediation as depicted in the lower part of Figure 6. The capsules containing the

especialized microorganisms can be placed in defined zones of the soil in such a way that a vertical "bioscreen" would be formed that intercepts a pollutant stream or plume. Simply by means of natural hydraulic gradients the target compounds are brought to the active layer where biodegradation takes place, provided that adequate environmental conditions (such as sufficient aeration, for instance) are given. The inoculated microorganisms would be therefore used in the most efficient way in a restricted zone with relatively defined conditions. Soil invasion, pumping and external treatment are reduced to a minimum. Such a system could be indicated for degradation of, for instance, halogenated or nitroaromatic and aliphatic pollutants. In theory, subsequent active layers could be used for degradation of diverse/complementary pollutants by inoculating different microorganisms/consortia and/or providing different environmental conditions (for instance aerobiosis/anaerobiosis).

## REFERENCES

Alexander, M. 1994. Biodegradation and Bioremediation. Academic Press, San Diego, California, USA.

Anid PJ, Nies L, Vogel T. 1991. Sequential anaerobic-aerobic biodegradation of PCB's in the river model. In Hinchee RE & Olfenbettel RF (Eds.) On-site bioreclamation: Processes of Xenobiotic and Hydrocarbon Treatment. Butterworth-Heinemann, Boston, pp. 428-436.

Armenante PM, Kafkewitz D, Lewandowski GA, Jou CJ. 1999. Anaerobic-aerobic treatment of halogenated phenolic compounds. Water Research 33: 681-692

Arnold FH. 1996. Directed evolution: Creating biocatalysts for the future.Chem. Eng. Sci 51:5091-5102

Arvin E, Scholtz-Muramatzu H, Janssen DB, Diekert G. 1995. Biological elimination of chlorinated ethylenes in combined anaerobic and aerobic biofilm systems (project no EV5V-CT92-0239). In: Workshop on projects awarded by the EC within the scope of the Environment & Climate Programme. Bilbao, Spain.

Bédard DL, Wagner RE, Brenna MJ, Haberl ML, Brown JF. 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environm. Microbiol. 53:1094-1102

Beunik, H., Rehm, H.J. 1988. Synchronous anaerobic and aerobic degradation of DDT by an immobilized mixed culture system. Appl. Microbiol. Biotech. 29:72-80.

Beunik H, Rehm HJ. 1990. Coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol by a co-immobilized mixed culture system. Appl. Micro. Biotech. 34:108-115.

Bosma T, Kruizinga E, de Bruin EJ, Poelarends GJ, Janssen DB . 1999. Utilization of trihalogenated propanes by Agrobacterium radiobacter AD1 through heterologous expression of the haloalkane dehalogenase from Rhodococcus sp strain m15-3. Appl. Environm. Microbiol. 65: 4575-4581

Chaudry GR, Chapalamadugu S. 1991. Biodegradation of halogenated organic compounds. Microbiol. Rev. 55:59-79

Beunik H, Rehm HJ (1990) Coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol by a co-immobilized mixed culture system. Appl Microbiol Biotechnol 34:108 115

Bosma T, Kruzinga E, Bruijn EJD, Poeklarends GJ, Janssen DB. 1999. Utilization of trihalogenated propanes by *Agrobacterium radiobacter* AD1 through heterologous exspression of the haloaljkane dehaklogenase from *Rhodococcus* sp. Strain M15-3. Appl. Environm. Microbiol. 65:4574-4581

Commandeur LCMH, Parsons JR. Biodegradation of halogenated aromatic compounds. In: Biochemistry of microbial degradation. Ratledge C (Ed.). 1993, 13:423-458

Criddle CS, McCarty PL. 1991. Electrolytic model system for reductive dehalogenation in aqueous environments. Environm. Sci. Technol. 25:1882-1889

Dalsgaard, T., de Zwart, J., Robertson, L.A., Kuenen, J.G., Revsbech, N.P. Nitrification, denitrification and growth in artificial *Thiosphaera pantothropha* biofilms as measured with a combined microsensor for oxygen and nitrous oxide. FEMS Microbiology Ecology, 1995, 17:137-148.

Daun, G, Lenke, H, Desiere, F, Stolpmann, H, Knackmuss, H-J. 1995 Biological treatment of TNT-contaminated soil by a two-stage anaerobic/aerobic process. In: Van den Brink *et al* (Eds.) Contaminated soil '95. Kluwer, Dordrecht, pp. 337-346.

Dickel O, Haug W, Knackmuss H-J. Biodegradation of nitrobenzene by a sequential anaerobic-aerobic process. Biodegradation, 1994, 4:187-194

de Beer, D., Van den Heuvel, J.C. Ottengraaf, S.P.P. 1993. Microelectrode measurements of activity distributions in nitrifying bacterial aggregates. Appl. Environm. Microbiol. 59:573-579

de Best JH, Salminen E, Doddema HJ, Janssen DB, Harder W. 1998. Transformation of carbon tetachloride under sulfate reducing conditions. Biodegradtaion 8:429-436

Duque, E. A. Haidour, F. Godoy, J.L. Ramos. 1993. Construction of a *Pseudomonas* hybrid strain that mineralizes 2,4,6-trinitrotoluene. J. Bacteriol. 175:8:2278-2283

Ensley DB. 1994. Designing pathways for environmental purposes.Curr. Opi. Biotechnol. 5:249-252

Fathepure, B.Z. Vogel, V.M. (1991). Complete degradation of polychlorinated hydrocarbons by a two-stage biofilm reactor. Appl. Environ. Microbiol. 57(12):3418-3422.

Fahmi M, Kut OM, Heinzle E. 1994. Anaerobic-aerobic fluidized bed biotreatment of sulphite pulp bleaching effluents – II. Fate of individual chlorophenolic compounds. Wat. Res. 28:1997-2010.

Field JA, Stams AJM, Kato M, Schraa G. Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consrtia. 1995. Antonie van Leeuwenhoek, 67:47-77

Fogel S, Lancione RL, Sewall AE. 1982. Enhanced biodegradation of methoxychlor in soil under sequential environmental conditions. Appl. Environm. Microbiol. 44:113-120

Funk SB, Roberts, DJ, Crawford DL, Crwaford RD. Initial-phase optimization for bioremediation of ammunition compound-contaminated soils. Appl. Environm. Microbiol. 1993, 59:2171-2177

Furukawa K, Hirose J, Hayashida S, Nakamura K. 1994. J. Bacteriol. 179:2121-2123

Gardin H., Pauss A. Co-immobilization of aerobic and anaerobic bacterial communities in a mixed k-carrageenan/gelatin gel for the degradation of 2,4,6-thrichlorophenol. 1994 *Proc. International Workshop Bioencapsulation IV*, Quebec, Canada, pp. 93-97

Gerritse J, Gottschal JC. 1992. Mineralization of the herbicide 2,3,6-thricholorobenzoic acid by a co-culture of anaerobic and aerobic bacteria. FEMS Microbiol. Ecol. 101:89-98

Gerritse J, Schut F, Gottschal JC. 1990. Mixed chemostat cultures of obligatory aerobic and fermentative or methanogenic bacteria grown under oxygen-limited conditions. FEMS Microbiol. Lett. 66:87-94

Gerritse J, Gottschal JC. 1993. Two-membered mixed cultures of methanogenic and aerobic bacteria under oxygen-limited conditions. J. Gen. Microbiol. 139:1853-1860

Gerritse J, Renard V, Visser J, Gottschal JC. 1995. Complete degradation of tetrachloroethene by combining anaerobic dechlorinating and aerobic methanotrophic enrichment cultures. Appl. Microbiol. Biotechnol. 43:920-928

Glasser A, Liebelt U, Hempel DC. 1992. Design of a two-stage process for total degradation of azo dyes. DECHEMA Biotechnol. Conf. 5:1085-1088

Goodall JL, Thomas SM, Spain JC, Peretti SW. 1998a. Operation of mixed-culture immobilized cell reactors for the metabolism of meta- and para-nitrobenzoate by Comamonas sp. JS46 and Comamonas sp.JS47, Biotechnol. Bioeng. 59 (1): 21-27

Goodall JL, Peretti SW. 1998a. Dynamic modeling of meta- and para-nitrobenzoate metabolism by a mixed co-immobilized culture of Comamonas spp. JS46 and JS47. Biotechnol. Bioeng. 59 : 507-516

Hakulinen R, Salkinoja-Salonen M. 1982. Treatment of pulp and paper industry wastewaters in an anaerobic fluidised bed reactor. Process Biochemistry17:18-22

Harayama S. 1998. Artificial evolution by DNA shuffling. Trends In Biotechnol., 16:76-82

Haug W, Schmidt A, Nortemann B, Hempel DC, Stolz A, Knackmuss H-J. 1990. Mineralization of the sulfonated azo dye Mordant Yellow 3 by a 6-aminonaphtalene-2-sulfonate-degrading bacterial consortium. Appl. Environm. Microbiol. 57:3144-3149

Havel J, Reineke W. 1991. Total degradation of variuos chlorobiphenyls by cocultures and in-vivo constructed hybrid pseudomonads. FEMS Microbiol. Lett. 78:163-170

Hellendoorn L,. Ottengraaf, S.P. Pennings, J.A.M.M., van den Heuvel, J.C., Martins dos Santos, V.A.P., Wijffels, RH. (1999). Kinetic Behaviour of and Performance of a Co-

immobilized System of Amyloglucosidase and *Zymomonas mmobilis*. Biotechnol. Bioeng. 63:694-704

Kato M, Field JA, Lettinga G. 1993. High tolerance of methanogens in granular sludge to oxygen. Biotechnol. Biotechnol. 42:1360-1366

Kafkewitz D, Armenante PM; Lewandowski G, Kung CM. 1992. Dehalogenation and mineralization of of 2,4,6-trichlorophenol by the sequential activity of aerobic and anaerobic microbial populations. Biotechnol. Lett. 14:143-148

Kettunen RH, Rintala JA. 1995. Sequential anaerobic –aerobic treatment of sulphur-rich phenolic leachates. J. Chem. Technol. Biotechnol. 62:177-184

Knackmuss H-J. 1996. Basic knowledge and perspectives of bioelimination of xenobiotic compounds. J. Biotechnol. 51:287-295

Kokufuta E, Shimohashi M, Nakamura I. Simultaneously occurring nitrification and denitrification under oxygen gradient by polyelectrolyte complex-coimmobilized *Nitrosomonas* and *Paracoccus denitrificans* cells. Biotechnol. Bioeng., 1988, 31:382-384.

Kokufuta E, Yukishige M, Nakamura I. Co-immobilization of *Nitrosomonas* and *Paracoccus denitrificans* cells polyelectrolyte complex-stabilized calcium alginate gel. J. Ferment. Bioeng. 1987; 6:659-664.

Kurosawa H, Tanaka H (1990) Advances in immobilized cell culture: development of a co-immobilized mixed culture system of aerobic and anaerobic micro-organisms. Process Biochem Int 25:189-196

Kotlar E., Tartakovsky B, Armagan Y, Sheintuch M. 1996. The nature of interaction between immobilized nitrification and denitrification bacteria. J. Biotechnol. 51:251-258

Kudlich M., Bishop PL, Knackmuss H-J, Stolz A. 1996. Simultaneous anaerobic and aerobic degradation of the sulfonated azo dye Mordant Yellow 3 by immobilized cells from a naphatlenesulfonate-degrading mixed culture. Appl. Microbiol. Biotechnol. 46:597-603

Lehrbach PR, Zeyer J, Reinenke W, Knackmuss H-J, Timmis KN. 1984. Enzyme recruitment in vitro: use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas* B13. J. Bacteriol. 158:1025-1032

Long D, Stensel DH. 1990. Proceedings Environmental Engineering Specialty Conference. O'Meia C (Ed.), Amer.Soc. Civil Eng. New York, pp. 183-190

Liu S, Suflita JM. (1993). Trends in Biotechnol. Ecology and evolution of microbial populations for bioremediation. 11:344-352.

Martins Dos Santos, V.A.P., Verschuren, P., Van Den Heuvel, H., Tramper, J., Wijffels, R. 2001a. Substrate and Product Profiles Across Double-Layer Gel Beads: Modelling and Experimental Evaluation. Biotechnology & Bioengineering, Submitted

Martins Dos Santos, V.A.P., Verschuren, P., Smit, M., Van Den Heuvel, H., Tramper, J., Wijffels, R. 2001b. pH Effects on Coupled Nitrification and Denitrification Measured by Specific Microelectrodes. Biotechnology & Bioengineering, Submitted

Martins Dos Santos, V.A.P., Tramper, J., M. Jetten, G. Kuenen R.H. Wijffels.2001c Simultaneous Autotrophic Nitrification and Anaerobic Ammonium Oxidation With Co-Immobilized Micro-Organisms. Appl. Environm Microbiol. Submitted

Martins Dos Santos, V.A.P., Tramper, J., M. Jetten, G. Kuenen R.H. Wijffels. 2001d. Design of a Fully Autotrophic Nitrogen Removal Process. Biotechnology & Bioengineering , Submitted.

Maymó-Gatell X, Chien Y-T, Gosset JM, Zinder SH. 199. Isolation of abacterium that reductively dechlorinates tetrachloroethene to ethene. Science, 276:1568-1571.

Muller R. 1992. Bacterial degradation of xenobiotics. In: Microbial control of pollution. JC Fry, GM Gadd, RA Herbert, CW Jones, IA Watson-Craick (Eds.) Symposium no. 48, Society for General Microbiology, Cambridge University Press, pp. 35-37.

Quensen-III JF, Tiedje JM, Boyd SA. 1988. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. Science. 242:752-754.

Peres CM, Naveau, H, Agathos, SN. Biodegradation of nitrobenzene by its simultaneously reduction into aniline and mineralization of the aniline formed. Appl. Microbiol. Biotechnol. 1998, 49:343-349

Peng CA, Bly MJ. 1998. Analysis of xenobiotic bioremediation in a co-immobilized mixed culture system. Biochemical Engineering Journal, 1: 63-75

Pieper DH, Reinecke W. 2000. Engineering bacteria for biotremediation. Current Opinion in Biotechnol. 11:262-270

Ramos, J.L., A. Wasserfallen, K. Rose, K.N. Timmis.1987. Redesigning metabolic routes: manipulation of the TOL plasmid pathway for catabolism of alkylbenzoates. Science 235:593-596.

Razo-Flores E, Lettinga G, Field JA. 1997. Biotransformation and biodegradation of selected nitroaromatics under anaerobic conditions Biotechnol. Progr. 15 :358-365.

Reinecke W. 1998. Development of hybrid strains for the mineralization of chloroaromatics by pathchwork asembly. Ann. Rev. Microbiol. 52:287-331

Rieger P-G, Knackmuss H-J. 1995. Basic knowledge and perspectives on biodegradation of 2,4,6-trinitrotoluene and related nitroaromatic compounds. In: Spain J. (Ed.) Biodegradation of nitroaromatic compounds. Plenum, New York. Pp. 1-18

Rojo, F., D.H. Pieper, K.H. Engesser, H.J.Knackmuss, K.N. Timmis. 1987. Assemblage of *ortho* cleavage route for simultaneous degradation of chloro- and methylaromatics. Science 238:1395-1398.

Semprini L. 1997. Strategies for the aerobic co-metabolism of chlorinated solvents. Curr. Opi. Biotechnol. 8:296-308

Shen CF, Guiot SR. 1996. Long-term impact of dissolved oxygen on the activity of anaerobic granules. Biotechnol. Bioeng. 49:611-620

Strous M, Fuerst JA, Kramer EHM, Logemann S, Muyzer G, van de Pas-Schoonen K, Webb R, Kuenen, JG, MJM Jetten. 1999. Missing lithotroph identified as new plactomycete. Nature 400,446-449

Tanaka H, Kurosawa H, Murakami H. 1986. Ethanol production from starch by a co-immobilized mixed culture system of *Aspergillus awamori* and *Zymomonas mobilis*. Biotechnol. Bioeng. 28:1761-1768

Tartakovsky B, Kotlar E, Sheintuch M. 1996. Coupled nitrification-denitrification processes in a mixed culture of co-immobilized cells:Analysis and experiments. Chem. Eng. Sci. 51:2327-2336

Tartakovsky B, Guiot SR, Sheintuch M. 1998. Modeling and analysis of co-immobilized aerobic/anaerobic mixed cultures. Biotechnol. Progr. 14:672-679

Tartakovsky B, Miguez CB, Petti L, Bourque D, Groleau D, Guiot SR. 1998. Tetrachloroethylene dechlorination usisng a consortium of co-immobilized methanogenic and methanotrophic bacteria. Enzyme Microbial Technol. 22:255-260

Tiedje JM, Sextone AJ, Parkin TB, Revsbech NP, Shelton DR. 1984. Anaerobic processes in soils. Plant soil 76:197-212

Timmis, KN, Steffan RF, Unterman R. 1994. Designing microorganisms for the treatment of toxic wastes. Annu. Rev. Microbiol. 48:525-557

Timmis, KN, pieper DH. 1999. Bacteria designed for biodegradation. Trends in Biotechnol. 17:201-204.

Tramper J (1984) Nitrification and denitrification by immobilized bacteria. In: Proceedings of the 3rd European Congress on Biotechnology, VCH Verlag, Weinheim, vol IV, pp 363 368

Wijffels RH, Eekhof MR, Van den Heuvel, JC, Tramper J. 1995. Pseudo-steady-state oxygen concentration profiles in an agar slab containing growing *Nitrobacter agilis*. J. Ferment. Bioeng. 2:167-170

Wilson GJ, Khodadoust AP, Suidan MT, Brenner RC. 1997. Anaerobic/aerobic biodegradation of pentachlorophenol using GAC fluidized be bioreactors. Wat. Sci. Technol. 36:107-115.

van de Graaf, A.A. A. Mulder, P. de Bruijn, M.S.M. Jetten, L.A. Robertson and J.G. Kuenen. 1996. Autotrophic growth of anaerobic ammonium oxidizing microorganisms in a fluidized bed reactor. Microbiology (UK). 142:2187-2196.

Van Eekert MHA, Schroder TJ, Stams AJM, Schraa G, Field J. Degradation of carbon tetrachloride in unadapted methanogenic granular sludge. Appl. Environm. Microbiol. 64:2350-2356

Van Hylckama Vlieg JET, de Koning W, Janssen DB. (1996) Transformation kinetcs of chlorinated ethenes by *Methylosinus trichosporium* OB3b and detection of unstable epoxides by on-line gas chromatography. Appl. Environ. Microbiol. 62:3304-3312

Zitomer DH, Speece RE. 1993. Sequential environments for enhanced biotransformations of aqueous contaminants. Environ. Sci. Technol. 27:227-244

## SUMMARY

Nitrogen compounds are commonly removed within wastewater systems by suspended or flocculated biomass (activated-sludge) in series of bioreactors. Due to the slow growth rate of the microorganisms involved (especially of those responsible for oxidation of ammonia and nitrite), long retention times and large aeration tanks are required for effective nitrogen removal. To meet the sharpened limit-values for total-nitrogen in wastestreams even larger treatment units are needed. Frequently the space necessary to realize these extensions is not available at the treatment site. In addition, although complete nitrogen removal can be achieved by coupling nitrification and denitrification, integration of these processes into a single system is in practice difficult to achieve because different environments are required by the nitrifying (strict aerobic) and denitrifying (facultative anaerobic) cells. Therefore, intensive research and development work has been done in the last decades to design, build and operate high-rate, compact reactor systems for nitrification and denitrification with minimal space needs whilst meeting the environmental requirements. However, despite of some progresses regarding compactness, in most of the improved systems developed so far, nitrification and denitrification are as yet carried out in separate reactors with some form of recirculation between them. These issues demanded thus further developments in the nitrogen-removal technology.

This thesis addresses the possibilities of coupling nitrification and denitrification in a compact reactor system by using co-immobilized mixed-cultures processes. This system relies on the establishment of oxic and anoxic niches within a single biocatalytic particle in which the relevant nitrifying and denitrifying bacteria are co-immobilized. The scope of this work, the main aims and the research outline are presented in **Chapter 1**.

**Chapter 2** gives an overview of the most recent advances on the development of compact systems for integrated nitrogen removal. First, a brief glance on the conventional configurations most commonly used is given. Compact systems based on immobilized-cell processes are then discussed on basis of their performance, adequacy and potential applicability at large scale. Novel nitrification-denitrification processes are evaluated as well as some important tools (such as modeling and its experimental evaluation by several ways) for the study of nitrogen removal in compact reactors. Finally, the future prospects on integrated nitrogen removal are discussed.

Subsequently, in **Chapter 3**, the Magic-Bead as an integrated approach for nitrification and denitrification is formally introduced. This chapter describes both qualitative and quantitative aspects of simultaneous autotrophic nitrification and heterotrophic denitrification by, respectively, the nitrifier *Nitrosomonas europaea* and either one of the denitrifiers *Pseudomonas denitrificans* or *Paracoccus denitrificans* co-immobilized in double-layer gel beads.

In **Chapter 4**, several methods are presented to produce double-layer beads for the selective co-immobilization of microbial cells. Coating, encapsulation and bead formation by a double-needle device are studied. Combinations of natural support materials such as carrageenan, alginate and chitosan, synthetic polymers such as polyvinyl alcohol and polyacrylonitrile, and pre-formed sintered glass, silica and celite beads, were investigated. The beads produced were evaluated with respect to their mechanical strength, ease of production, shape and mildness for cell immobilization.

The mechanical stability of biocatalyst particles in bioreactors is of crucial importance for applications of immobilized-cell technology in bioconversions. The common methods for evaluation of the strength of polymer beads (mostly force-to-fracture or tensile tests) are, however, not yet proven to be relevant for the assessment of their mechanical stability in bioreactors. Therefore, in **Chapter 5**, the relevance of these properties for abrasion of gel beads in bioreactors is investigated on the basis of a rigorous approach to evaluate both abrasion and rheological properties.

The use of immobilized-cell processes for the treatment of wastewater would require the carrier material to meet criteria that would guarantee a certain effectiveness and efficiency. Therefore in **Chapter 6** the characteristics of several natural and synthetic materials are determined and compared to each other so to establish a rational strategy to choose the most suitable support materials for a given process.

**In Chapter 7** a mechanistic dynamic model describing integrated nitrification and denitrification by *Nitrosomonas europaea and Pseudomonas* spp. co-immobilized in the separate layers of double-layered gel beads is presented. The model describes diffusion of components, substrate utilization, and growth, all occurring simultaneously in the beads. It predicts biomass and solute bulk concentrations, substrate consumption rates, product formation rates, and biomass and solute concentration profiles within the beads as a function of time. Fluctuations in substrate load, dilution rates, or mass transfer parameters can be accommodated as well. The model was evaluated under both steady

and dynamic operating conditions by comparing experimental and predicted bulk concentrations and macroscopic consumption (production) rates in air-lift loop reactors containing double-layer gel beads.

An in-depth investigation of the steady and transient behavior of the proposed system at biocatalyst level is done in **Chapter 8** by coupling detailed model predictions with accurate experimental measurements of local solute concentrations under a wide range of conditions. The $O_2$, $NH_4^+$, $NO_2^-$ gradients across a double-layered slab with co-immobilized *Nitrosomonas europaea* and *Pseudomonas spp.* were measured regularly with microelectrodes and compared to model simulations under nitrifying, denitrifying and coupled nitrifying and denitrifying conditions. Under all circumstances the predicted microprofiles matched well the gradients measured experimentally. Also, the transitions from one set of conditions to the other were in all cases properly accounted for by the model. This work is the first report on a detailed study of a coupled oxidative-reductive processes by co-immobilized bacteria that is supported by both a sound theoretical framework and an accurate experimental technique to describe these processes at biocatalyst level.

Despite its enormous importance on nitrogen removal by immobilized-cell processes, the knowledge of the complex interdependencies involved in the generation of pH gradients within biocatalytic supports and of their effect on (de)nitrification is still very limited. Hence, in **Chapter 9** the dynamic model developed earlier is extended to account for the development of pH profiles across the biocatalysts, including the buffering effects of the medium components as well as those resulting from conversion products. Model predictions were compared successfully with the experimental profiles measured with pH, $O_2$, $NH_4^+$ and $NO_2^-$ microelectrodes for nitrifying, denitrifying and coupled nitrifying-denitrifying conditions.

On the basis of the "Magic-Bead Concept" (Figure 1), in **Chapter 10** a compact, fully autotrophic single-staged system is proposed to combine "classical" aerobic nitrification by autotrophic nitrifying cells with the recently discovered anaerobic ammonium oxidation process (Anammox). In this combined process, the ammonium supplied to the reactor medium is partly oxidized by nitrifying cells immobilized in the outer layer of the beads. Due to the fast oxygen uptake by the nitrifiers, both biomass and substrate profiles develop leading to the establishment of extensive anoxic zones within the beads. The nitrite produced by the aerobic nitrifiers and the remaining ammonium are then anaerobically converted into nitrogen by the Anammox microorganisms entrapped

in the bead core. The system was shown to work both in batch and in continuous mode. This is the first report of a single-staged, fully autotrophic system capable of converting ammonia to dinitrogen gas without the need of an external electron acceptor other than oxygen.

To gain insight into the mechanisms underlying the processes involved in this autotrophic arrangement and to be able to design further applications of the system, in **Chapter 11** a dynamic model describing the interactions among nitrifying (*Nitrosomonas* spp. *and Nitrobacter* spp.) and Anammox microorganisms co-immobilized within single-layered gel beads was developed on the basis of the above described model. The model predicted adequately the experimentally measured behavior of the system (growth, conversion of substrates and mass transport) both under steady and dynamic conditions. The proposed system is highly compact and reduces greatly the energy and organic-C inputs. Therefore, it may be a valuable alternative for effective nitrogen removal from wastewaters.

After having studied in detail several of the most relevant issues regarding the "Magic Bead Concept" in chapters 2 through 11, in **Chapter 12** the possibilities of integrating many other oxidative and reductive complementary biodegradation processes in compact systems by using co-immobilized mixed-culture systems are addressed. Relying on this view, the perspectives on the biodegradation of recalcitrant compounds as well as their implications for in-situ and on-site bioremediation of contaminated sites are discussed.

## SAMENVATTING

In waterzuiveringsprocessen worden stikstofverbindingen in het algemeen verwijderd met behulp van gesuspendeerde biomassa (actief slib) in geschakelde bioreactoren. Vanwege de lage groeisnelheden van de betreffende micro-organismen (en met name diegene die ammoniak en nitriet oxideren) moet het afvalwater lang in grote bioreactoren verblijven om uitspoeling van bacteriën te voorkomen. Om de steeds strengere lozingseisen voor stikstofverbindingen te kunnen bereiken moeten grotere en meerdere geschakelde reactoren gebruikt worden. Theoretisch is het mogelijk om in een systeem alle stikstofverbindingen te verwijderen door gekoppelde nitrificatie en denitrificatie, maar in de praktijk blijkt het zeer moeilijk om beide processen te integreren omdat nitrificerende micro-organismen zuurstof nodig hebben voor het verwijderen van stikstofverbindingen en denitrificeerders juist niet. Om deze redenen wordt er veel onderzoek gedaan naar het ontwerp van compacte reactorsystemen die een hoge verwijderingsnelheden kunnen bereiken en derhalve weinig ruimte vragen. Alhoewel er compactere systemen zijn ontworpen, worden nitrificatie en denitrificatie vooralsnog in gescheiden reactoren uitgevoerd die met elkaar in verbinding staan via een recirculatiesysteem. Tot dusverre ontbreekt een werkelijk compact en geïntegreerd reactorsysteem.

In dit proefschrift worden de mogelijkheden onderzocht om nitrificatie en denitrificatie te combineren in een compact reactorsysteem door gebruik te maken van geïmmobiliseerde mengculturen van nitrificerende en denitrificerende micro-organismen. Het systeem is gebaseerd op het ontstaan van oxische en anoxische zones in één enkel biokatalytisch deeltje met respectievelijk geïmmobiliseerde nitrificerende en denitrificerende bacteriën. De doelen, omvang en hoofdpunten van dit onderzoek worden in **Hoofdstuk 1** gepresenteerd.

In **Hoofdstuk 2** wordt een overzicht gegeven van recente vooruitgangen in de ontwikkeling van compacte systemen voor geïntegreerde stikstofverwijdering. Compacte systemen gebaseerd op geïmmobiliseerde cellen worden behandeld aan de hand van de capaciteit, geschiktheid en potentieel voor grootschalige toepasbaarheid. Nieuwe nitrificatie-denitrificatie processen worden beoordeeld en relevante methoden om stikstofverwijdering in bioreactoren te bestuderen (zoals modellen) bediscussieerd.

In **Hoofdstuk 3** wordt "de Toverbal" als geïntegreerd concept voor gecombineerde nitrificatie-denitrificatie geïntroduceerd. Het hoofdstuk beschrijft kwalitatieve en

kwantitatieve aspecten van simultane autotrofe nitrificatie en heterotrofe denitrificatie door gelaagde co-immobilisatie van de nitrificeerder *Nitrosomonas europaea* en een denitrificeerder, *Paracoccus denitrificans* danwel *Pseudomonas denitrificans*, in een gelbolletje.

**Hoofdstuk 4** beschrijft verschillende methoden voor de productie van tweelagige bolletjes voor de selectieve co-immobilisatie van de nitrificerende en denitrificerende bacteriën. Coating, insluiting en bolvorming door een dubbelnaalds druppelaar worden bestudeerd. Combinaties van natuurlijk dragermaterialen als carrageen, alginaat en chitosan, synthetische materialen als polyvinylalcohol en polyacrylonitriel en bolletjes van gesinterd glas, silica en celiet zijn onderzocht. De geproduceerde bolletjes zijn vervolgens beoordeeld op mechanische sterkte, eenvoud van het productieproces en mildheid voor immobilisatie.

De mechanische stabiliteit van de biokatalysatordeeltjes in bioreactoren is zeer belangrijk voor de toepassing van geïmmobiliseerde cellen in bioconversies. Van de gangbare methoden om de sterkte van polymeerdeeltjes te meten (met name sterkte ten opzichte van breuk) is nog niet aangetoond dat ze bruikbaar zijn om hun mechanische stabiliteit in bioreactoren te beoordelen. In **Hoofdstuk 5** wordt de stabiliteit van deeltjes in bioreactoren onderzocht. Hierbij wordt onder andere gekeken naar de breukeigenschappen van een gel in relatie tot de slijtagegevoeligheid van een gelbolletje in een bioreactor.

Voor toepassing van geïmmobiliseerd cellen in afvalwaterzuiveringsprocessen worden eisen gesteld met betrekking tot effectiviteit en efficiëntie. Daarom zijn in **Hoofstuk 6** de eigenschappen van een aantal natuurlijke en synthetische materialen bepaald en met elkaar vergeleken. Hiermee kan voor een bepaald proces de meest geschikte drager worden gekozen.

In **Hoofdstuk 7** wordt een mechanistisch dynamisch model voor geïntegreerde nitrificatie en denitrificatie in een tweelagig gelbolletje gepresenteerd. De buitenste laag van het bolletje bestaat uit geïmmobiliseerde nitrificerende bacteriën (*Nitrosomonas europaea*) en de kern uit geïmmobiliseerde denitrificeerders (*Pseudomonas spp.*) Het model bevat informatie over stofoverdracht, kinetiek van de micro-organismen en informatie over hoe de bioreactor bedreven wordt. Zo kunnen veranderingen in substraatconcentratie, verdunningssnelheid of stofoverdracht ingebracht worden. Het model voorspelt biomassa- en stofconcentraties in de bulk en productiesnelheden en

concentratieprofielen in het deeltje zelf. Het model is experimenteel geëvalueerd in een airlift reactor met geïmmobiliseerde cellen. Hiervoor zijn gemeten en voorspelde bulkconcentraties en macroscopische productiesnelheden met elkaar vergeleken onder statische en dynamische omstandigheden.

In **Hoofdstuk 8** wordt het effect van statische en dynamische omstandigheden op het biokatalysatordeeltje onderzocht. In een tweelagige plaat met daarin geco-ïmmobiliseerde *Nitrosomonas europaea* en *Pseudomonas spp.* cellen zijn onder verschillende condities de gemeten zuurstof-, ammonium- en nitrietprofielen in de plaat vergeleken met modelvoorspellingen. Onder alle omstandigheden bleken de voorspellingen goed overeen te komen met de experimentele waarden. Dit werk is de eerste gedetailleerde studie op deeltjesniveau waarbij een gekoppeld oxidatie-reductie proces met behulp van geïmmobiliseerde bacteriën gesteund wordt door een solide theoretische ondergrond in combinatie met een nauwkeurige experimentele techniek.

Ondanks het grote belang van stikstofverwijdering met behulp van geïmmobiliseerde cellen is het begrip over de complexe samenhang tussen het ontstaan van pH-gradiënten in het biokatalytische deeltje en het effect op het (de)nitrificatieproces zeer beperkt. Daarom wordt in **Hoofdstuk 9** het dynamische model uitgebreid om de ontwikkeling van pH-gradiënten in het deeltje te kunnen beschrijven. In het model wordt het bufferende effect van het medium door de diverse bioconversies meegenomen. De met microelectroden gemeten pH-, zuurstof-, ammonium- en nitrietprofielen kwamen goed overeen met de modelvoorspellingen.

Op de basis van het "Toverbal-Concept" wordt in **Hoofdstuk 10** een compact en volledige autotroof enkel-traps systeem voorgesteld. "Klassieke" aërobe nitrificatie door autotrofe nitrificerende cellen wordt gecombineerd met een recentelijk ontdekt, anaëroob ammoniumoxidatieproces (Anammox). Ammonium wordt gedeeltelijke geoxideerde door de nitrificeerders in de buitenste laag van de bolletjes. Als gevolg van de snelle zuurstofopname door de nitrificeerders ontwikkelt zich een steil biomassa- en substraatprofielen aan de rand van het deeltje en ontstaat er een uitgestrekte anoxische zone in het centrum van het deeltje. In deze zone worden het nitriet, gevormd door de nitrificeerders, en het overgebleven ammonium in afwezigheid van zuurstof omgezet naar stikstof. De werking van het systeem is getest in een batch en continue proces.

Om inzicht te krijgen in de onderliggende mechanismen van het enkel-traps proces en om toekomstige toepassingen met dit systeem te kunnen ontwerpen is in **Hoofdstuk 11**

een dynamisch model (gebaseerd op het hierboven beschreven model) gegeven. Het model beschrijft de interacties tussen de in een bolletje geïmmobiliseerde nitrificeerders (*Nitrosomonas* spp. en *Nitrobacter* spp.) en Anammox micro-organismen. Het model bleek het gedrag van het systeem (zoals groei, substraat omzetting en stofoverdracht) goed te voorspellen onder statische en dynamische omstandigheden. Het voorgesteld systeem is zeer compact en vraagt minder input van energie- en koolstofbronnen. Derhalve, is het systeem mogelijk waardevol alternatief om stikstof uit afvalwater te verwijderen.

Na de gedetailleerde studie van een aantal relevante factoren met betrekking tot het "Toverbal-Concept" in de hoofdstukken 2 tot 11, wordt in **Hoofdstuk 12** de mogelijkheid besproken om ook andere oxidatie-reductie processen te reduceren tot compacte systemen door gebruik te maken van geïmmobiliseerde mengculturen. De implicaties van dit concept voor *in-situ* and *on-site* sanering van gecontamineerde bodem wordt bediscussieerd.

## LIST OF SCIENTIFIC CONTRIBUTIONS

### Publications in peer-reviewed scientific journals and book chapters

**Martins dos Santos, V.A.P., Tramper, J. Wijffels, R.H.** (1992). Integrated Nitrification and Denitrification with Immobilized Bacteria, in Biofilms - Science and Technology, L.F. Melo, T.R. Bott, M. Fletcher and B. Capdeville (Eds.), Nato Series, pp. 449-453.

**Martins dos Santos V.A.P., Tramper, J., Wijffels R.H.** (1993). Simultaneous Nitrification and Denitrification Using Immobilized Microorganisms, Biomat. Artif. Cells & Immob. Biotech., 21: 317-322.

**Wijffels R.H., Martins dos Santos, V.A.P., Tramper, J.** (1993). Geintegreerd Nitrificatie en Denitrificatie met Behulp van Geimmobiliseerde Microorganismen: het Toverbal Concept. Procestecnologie,12:17-22

**Leenen, E.J.T.M., Martins dos Santos, V.A.P.M, Tramper, J., Wijffels, R.H.** (1994). Stability of Carrier Materials for (Waste) Water Treatment. ACS Polymer Preprints. 35(2), 81-82.

**Martins dos Santos, V.A.P., L. Marchal, Tramper, J., R.H. Wijffels.** (1996). Modelling and Evaluation of an Integrated Nitrogen Removal System with Co-immobilized Microorganisms. Biotechnol. Prog. 12:163-171

**Leenen E.J.T.M., V.A.P. Martins dos Santos, J. Tramper, R.H. Wijffels.** (1996). Characteristics and Selection Criteria of Support Materials for Immobilization of Nitrifying Bacteria. Progr. Biotechnol. 11: 205-212.

**Martins dos Santos, V.A.P., Tramper, J., R.H. Wijffels.** (1996) The Magic Bead Concept: An Integrated Approach for Nitrogen Removal with Co-immobilized Microorganisms. Appl. Microbiol. Biotechnol. 45:447-453.

**Martins dos Santos, V.AP.M., Tramper, J., Wijffels, R.H.** (1996). Dynamic Modelling of an Integrated Nitrogen Removal System with Co-immobilized Cells. Progr. Biotechnol. 11: 496-493.

**Leenen, E.J.T.M., Martins dos Santos, V.A.P., Grolle, K., Tramper, J. and R.H. Wijffels.** 1996. Characterization and Selection Criteria of Support Materials for Cell Immobilization. Water Research 30:2895-2996.

**Martins dos Santos, V.A.P., Vasilevska T., Kajuk, B., Tramper, J and R.H. Wijffels.** (1997). Production and Characterization of Double-Layer Beads for Co-immobilization of Microbial Cells. Biotechnology Annual Review, 3: 227-244

**Martins dos Santos, V.A.P., Leenen, E.J.T.M., van Vliet, T., J.Tramper, R.H.Wijffels.** (1997). Relevance of the Rheological Properties of Gel Beads for Their Mechanical Stability in Bioreactors. Biotechnology & Bioengineering. 56: 217-229

**Gódia, F., Martins dos Santos, V.A.P., Poncelet, D.** (1997). Editors of the Proceedings of the International Workshop on Bioencapsulation VI - From Fundamentals to Industrial Applications.Universidad Autónoma de Barcelona, Spain, 326 pp.

**Martins dos Santos, V.A.P., J-L. Ramos.** (1997) Biodegradation of Aromatic Hydrocarbon Mixtures in Heavily Contaminated Streams by Immobilized Recombinant Microorganisms. In: B.Alleman, A. Leeson (Eds.). In-situ and On-site Bioremediation: Volume 4. Battelle PressColumbus, Ohio, USA, PP. 56-57

**Martins dos Santos, V.A.P., Tramper, J., R.H. Wijffels.** (1998) Integrated Nitrogen Removal in Compact Systems: New Generation Bioreactors. Biotechnology Annual Review. 4:321-394

**Hellendoorn L,. Ottengraaf, S.P. Pennings, J.A.M.M., van den Heuvel, J.C., Martins dos Santos, V.A.P., Wijffels, RH.** (1999). Kinetic Behaviour of and Performance of a Co-immobilized System of Amyloglucosidase and *Zymomonas mmobilis*. Biotechnology and Bioengineering. 63:694-704

**Garbayo I, Vigara, AJ, Conchon V, Martins dos Santos VAP, Vílchez, C.** (2000). Characterization of Nitrate Consumption Of Immobilized *Chlamydomonas* Cells. Process Biochemistry 36:459-466.

**Martins Dos Santos VAP.** (2001) *In-situ* bioremediation of heavily contaminated groundwaters by specialized-microorganisms using bioscreens. In: Evaluation of Demonstrated and Emerging Remedial Action Technologies for the Treatment of Contaminated Land and Ground Water. Kovalick W, James S. (Eds.) EPA, Cincinnati. USA. In press.

**Martins Dos Santos, V.A.P., Verschuren, P., Van Den Heuvel, H., Tramper, J., Wijffels, R.** Substrate And Product Profiles Across Double-Layer Gel Beads: Modelling And Experimental Evaluation. Biotechnology & Bioengineering, Submitted.

**Martins Dos Santos, V.A.P., Verschuren, P., Smit, M., Van Den Heuvel, H., Tramper, J., Wijffels, R.** pH Effects On Coupled Nitrification And Denitrification Measured By Specific Microelectrodes. Biotechnology & Bioengineering, Submitted.

**Lorite, M. J., Martins Dos Santos, V.A.P, Bedmar, E.** Growth Kinetics of *B. Japonicum* for Efficient CO Removal. Appl. Microbiol. Biotechnol, Submitted.

**Martins Dos Santos, V.A.P., Tramper, J., R.H. Wijffels.** Towards An Integrated Approach For Oxidative And Reductive Biodegradation Processes. Submitted.

**Huertas, M-J., Ramos, J.L. Martins Dos Santos, V.A.P.** Simulation of Solvent Discharge Into Soils In A Column Microcosms. Appl. Environm Microbiol. Submitted.

**Martins Dos Santos, V.A.P., Tramper, J., M. Jetten, G. Kuenen R.H. Wijffels.** Simultaneous Autotrophic Nitrification And Anaerobic Ammonium Oxidation With Co-Immobilized Micro-Organisms. Appl. Environm Microbiol. Submitted.

**Martins Dos Santos, V.A.P., Tramper, J., M. Jetten, G. Kuenen R.H. Wijffels.** Design Of A Fully Autotrophic Nitrogen Removal Process. Biotechnology & Bioengineering , Submitted.

**Martins Dos Santos, V.A.P., Weber J, Hecht V., Timmis KN.** Metabolic Model Of A 4-Chlorosalycilate Degrading *Pseudomonas putida* Strain. Submitted

**Martins Dos Santos, V.A.P. , Weber J, Hecht V., Timmis KN.** Understanding Metabolic Networking In Microbial Consortia Degrading Xenobiotics. In Preparation

**Martins Dos Santos, V.A.P., Hunik, J., Tramper, J., R.H. Wijffels.** Kinetics Of Double-Substrate Limitation By *Nitrosomonas Europaea*. In Preparation.

**Martins Dos Santos, V.A.P., Ramos, J.L.** Biodegradation Kinetics Of BTEX Mixtures At High Concentrations By Pseudomonas Putida DOT-T1, A Solvent-Tolerant Microorganism. In Preparation.

**Huertas, M-J., Ramos, J.L. Martins Dos Santos, V.A.P.** Simulation Of A Gasoline Spill Onto Soils In A Column Microcosms. In Preparation

## Proceedings and abstracts

**Martins Dos Santos, V.A.P., Lapa, R.S., Monteiro, M.J., Costa Lima, J.L** (1990). Determinação Automatica De Sulfatos Em Vinhos Do Porto Por Condutimetria. Proc. IV Encontro Luso-Galaico De Quimica, Vigo, Spain.

**Martins Dos Santos, V.A.P., Hunik, J., Tramper, J.** (1991). Nitrification Within Manure Treatment, Abstract Book Of The Workshop On Industrial Wastes Treatment, Instituto Superior Técnico, 27 September Lisboa, Portugal;

**Martins Dos Santos, V.AP.M., Tramper, J., Wijffels, R.H.** (1992). Integrated Nitrification And Denitrification With Coimmobilized Microorganisms', Proceedings Of The Congress "Bioencapsulation, A Key To The Bioindustries. Cachan, France.

**Martins Dos Santos V.A.P., Tramper, J, Wijffels, R.H.**(1993). Coupled Nitrification And Denitrification: The Magic Bead Concept, Proceedings Of The Sixth European Congress Of Biotechnology (Abst. MO189), Florence, Italy.

**Martins Dos Santos V.A.P., Tramper J., Wijffels, R.H..** (1993) Integrated Nitrification And Denitrification With Immobilized Microorganims. OSPT - Procestechnologie, Technical University Twente, Pp-265.

**Martins Dos Santos V.A.P., Tramper J., Wijffels, R.H.** (1993), Nitrogen Removal With Immobilized Microorganisms Abstract Book Of The Workshop Bioencapsulation Research Group, 19-21 October, Brussels, Belgium.

**Martins Dos Santos V.A.P., Tramper J., Wijffels, R.H.** (1994). (De)Nitrification Of Wastewater With Immobilized Microorganisms, Proc. Fifth Netherlands Congress On Biotechnology, 2-3 February, Amsterdam.PJ-33.

**Martins Dos Santos V.A.P., Tramper J., Wijffels, R.H. (1994).** (De-)Nitrification With Immobilized Microorganims, Proc. 27th FNK European Conference On Potato Processing, November 10-12 1993, Doorwerth, The Netherlands

**Martins Dos Santos V.A.P., Tramper J., Wijffels, R.H..** (1995) Dynamic Modelling Of A Combined Nitrogen Removal Using Co-Immobilized Microorganims. OSPT -

Procestechnologie, Technical University Twente, Pp-276. Mention With Honours For The Corresponding Poster.

**Leenen E.J.T.M., V.A.P. Martins Dos Santos, J. Tramper, R.H.** Wijffels. (1995). Characteristics And Selection Criteria Of Support Materials For Immobilization Of Nitrifying Bacteria. Abstract Book Of The International Symposium On Immobilized Cells: Basics And Applications. Noorderwijkerhout, The Netherlands, November

**Martins Dos Santos, V.AP.M., Tramper, J., Wijffels, R.H.** (1996). Dynamic Modelling Of An Integrated Nitrogen Removal System With Co-Immobilized Cells. Abstract Book Of The International Symposium On Immobilized Cells: Basics And Applications. Noorderwijkerhout, The Netherlands, November 1995

**Martins Dos Santos V.A.P., Tramper J., Wijffels, R.H.**. (1996). Towards An Integrated Approach For Oxidative And Reductive Biodegradation Processes. Case Study: Integrated Nitrogen Removal With Co-Immobilized Microorganisms. Proceedings Of The Symposium On Biodegradation Of Organic Compounds, June, Palma De Mallorca, Spain.

**Martins Dos Santos, V.A.P., Verschuren, P.G., Tramper, J., Wijffels, R.H.** (1996)An Integrated Approach For Nitrogen Removal: Modeling And Experimental Validation. In: Proceedings Of The Third Iberic Congress On Biotechnology, BIOTEC'96. Férnadez-Polanco, F, Garcia-Encina, P., González-Benito, G., Peña-Miranda, M. (Eds.) Universidad De Valladolid, Pp. 161-164.

**Martins Dos Santos, V.A.P., Ramos, J.L.** (1997) Biodegradation Of Aromatic Hydrocarbon Mixtures In Heavily Contaminated Streams By Immobilized Recombinant Microorganisms. Proceedings Of The International Workshop On Bioencapsulation VI - From Fundamentals To Industrial Applications. Gódia, F., Martins Dos Santos, V.A.P., Poncelet, D. (Eds). Universidad Autónoma De Barcelona, 326 Pp.

**Godoy, P., Duque, E., Mosqueda, G., Huertas, M-J., Alaminos, M., Martins Dos Santos, V.A.P., J.-J. Rodriguez-Herva, Ramos, J-L-.Ramos.** (1997). Isolation and Characterization Of The *Pseudomonas Putida* DOT-T1 Which Grows and Metabolizes Toluene and Other Aromatic Hydrocarbons At High Concentrations. Proceeding Of The 4th International Congress On Pseudomonas, Madrid, 4-7 September 1997.

**Martins Dos Santos, V.A.P., Huertas, M-J., Duque, E., Mosqueda, G., Segura, A., Ramos, JL.** (1998) Solvent Resistant Bacteria For Effective Bioremediation. Case Study: Biodegradation Of Aromatic Hydrocarbon Mixtures In Heavily Contaminated Groundwaters And Soils By Immobilized Pseudomonas Putida DOT-T1. Presented At The I Ibero- American Congress On Biotechnology, Held In Guimaraes, Portugal, July. PP 219-220

**Fernández-Sierra, ML García-Chaves., MA, Martins Dos Santos, VAP, Bedmar.JE.** (2000). Recombinant *Radyrhizobium Japonicum* Strains With Improved Efficiency In Removal Of Nitrite Under Microaerobiosis. IV European Conference On Nitrogen Fixation. 16-20 September, Seville, Spain.

**Martins Dos Santos, VAP, KN Timmis** (2000). Understanding Metabolic Networks In Microbial Consortia By Flux Balancing And Kinetic Modelling. Conference "Metabolic Engineering III", Colorado Springs, USA, 22-27 October

## *CURRICULUM VITAE*

Vítor Alexandre Pires Martins dos Santos, born 15 February 1968, Lisbon, Portugal.

1987-1992 College of Biotechnology (ESB), Portuguese Catholic University, Porto, Portugal. Five-year Food Engineering programme concluded in February 1992 with an overall grade of 16.3/20.0 (Honours).

1992-1996 Food and Bioprocess Engineering Group, Wageningen Agricultural University, The Netherlands. Ph.D. research project in the field of Environmental Bioprocess Engineering. Fellowship by JNICT, Portugal. Supervision: Prof. Johannes Tramper and Dr. Rene Wijffels.

1996-1997 Postdoctoral fellow at the Spanish National Scientific Research Board (CSIC), Estación Experimental del Zaídin, Department of Biochemistry. Fellowship by the EERO (European Environmental Research Organization). Supervision: Dr. Juan Luís Ramos.

1997-1999 Research Associate at the Spanish National Scientific Research Board (CSIC), Estación Experimental del Zaídin, Department of Biochemistry. Funded by the EU (RTD – Environment & Climate).

1999- Research Associate at the Department of Microbiology of the German Research Centre for Biotechnology (GBF), Braunschweig, Germany. Funded by the European Science Foundation, Gulbenkian Foundation and GBF itself. Supervision: Prof. Dr. Kenneth Timmis