NITRIFICATION BY ARTIFICIALLY IMMOBILIZED CELLS

Model and practical system

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Nitrification by artificially immobilized cells
Model and practical system

Proefschrift
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op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 2 april 1996
des namiddags te 16.00 uur in de Aula

Ter nagedachtenis aan mijn Oma,
wat was ze er graag bijgeweest.

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STELLINGEN

1. Een proces met geïmmobiliseerde (nitrificerende) cellen is geschikt als modelsysteem, maar heeft ook goede toepassingsmogelijkheden in de praktijk.
   dit proefschrift

2. Experimenten waarbij herhaaldelijk compressies worden uitgevoerd op een hydrogel geven een betere indicatie van de slijtagegevoeligheid (c.q. materiaalmoeheid) van dit gel *(dit proefschrift)* dan het meten van de breuksterkte, breukweerstand, vervorming, breukenergie of elasticiteitsmodulus.
   Muscat A, Beyersdorff J, Vorlop KD (1993) Biotechnol. Techn. 7: 591-596;
   Smidsrød O, Christensen BE (1991) TIBTECH 8: 71-78;
   Sumino T, Nakamura H, Mori N, Kawaguchi Y (1992) Appl. Microbiol. Biotechnol. 36: 556-560.

3. Fundamenteel onderzoek is een rekbaar begrip, dat door wetenschappers anders geïnterpreteerd wordt dan door mensen in de praktijk.

4. De specifieke ATP-niveaus in cellen, zoals gemeten door Gikas en Livingston (1993), zijn niet geschikt als indicator voor de levensvatbaarheid van geïmmobiliseerde cellen.
   Gikas P, Livingston AG (1993) Biotechnol. Bioeng. 42: 1337-1351.

5. Door de vele dierziektes voorkomend in de bioindustrie, zoals BSE en de varkenspest, wordt het gemakkelijk om uit te leggen waarom iemand geen vlees eet.

6. Bio(proces)technologie is niet per definitie hetzelfde als moleculaire biologie of genetica en omvat dus meer dan het gebruik van genetisch gemodificeerd materiaal.

7. Als 1 schaap gekloneerd is, is het hek van de dam.

8. Door het spelen van toneel leert men zichzelf en zijn omgeving goed kennen.

9. Vrouwelijke wetenschappers worden nog steeds niet altijd voor vol aangezien tijdens congressen, getuige de verkleinwoordjes die aan hun adres worden geuit.

   zoals: Moeilijk hè, mevrouwtje ! (Sewage 2000, 1992)

10. Het is aan te raden om studenten te verplichten gedurende hun studie ten minste één stage in een andere cultuur te volbrengen.

11. De kunst van het schrijven van wetenschappelijke publicaties is het weglaten.

12. Als aan proceskundigen gevraagd wordt een optimale route te volgen, zijn ze meestal te eigenwijs om deze te gebruiken.

    Buitenlandse reizen sectie Proceskunde

Stellingen behorende bij het proefschrift: 'Nitrification by artificially immobilized cells, model and practical system'.

E.J.T.M. Leenen
Wageningen, 2 april 1997

VOORWOORD

Dit proefschrift zou niet tot stand gekomen zijn zonder alle steun en vertrouwen van René Wijffels en Hans Tramper. Hun kritische blik bracht vaak de hoofdlijnen van het onderzoek en artikelen weer helder voor ogen. Heel erg bedankt voor de prettige samenwerking en alle mogelijkheden die jullie me geboden hebben.

Tijdens mijn onderzoek hebben vele personen uit binnen- en buitenland een waardevolle bijdrage geleverd aan het tot stand komen van dit proefschrift. Bedankt hiervoor: Åse Bakketun, Anja Boogert, Ana Teresa da Silva Ferreira, Stefan de Graaf, Gijs Driessen, Hiroki Emori, Gerard Engbers, Grazyna Englund, Marijke Ferdinandy, Teresa Fernandes, Ad Heide, Kees de Gooijer, Katja Grolle, Ida Günther, Simone Houtman, Jan Hunik, Léon Jansen, Lars Koens, Chris Lammers, Alette Langenhoff, Vitor Martins dos Santos, Karin Marquering, Andreas Muscat, José Obón de Castro, Hiro Nakamura, Mireia Rippol Martinez, Pieter Stamperius, Anne van Boxtel, Fred van den End, Sander van der Hark, Catrinus van der Sluis, Cheryl van der Kaa, Arie van der Vlies, André van Lammeren, Mark van Loosdrecht, Ton van Vliet, Marian Vermüe, Klaus Vorlop en alle ex-collega's van de sectie Proceskunde, werkplaats en fotolocatie.

Mijn ouders wil ik bedanken voor alle kansen die ik van hen kreeg en hun nimmer aflatende belangstelling ('kunnen we niet helpen ?').

Frank, bedankt voor jezelf!

CONTENTS

CHAPTER 1

NITRIFICATION BY ARTIFICIALLY IMMOBILIZED CELLS
Dynamics, supports and applications

Studies on the complex interactions in immobilized-cell systems have been carried out in our group for more than a decade. Nitrification by cells entrapped in carrageenan was used as a model system. Nitrification is the oxidation of ammonia via nitrite to nitrate:

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O$$
$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$$

Nitrosomonas europaea is responsible for the conversion of ammonia into nitrite and *Nitrobacter agilis* for the conversion of nitrite into nitrate. These autotrophic microorganisms have, under optimal conditions, a generation time of about 20 hours.

By immobilization microorganisms can be retained at high concentrations in a reactor. This can be done by crosslinking of cells, microencapsulation, covalent bonding to a support, natural attachment, or entrapment in a polymer matrix. In our studies this was done by entrapment in alginate or carrageenan (Hunik *et al.*, 1994; Tramper and Grootjen, 1986; Van Ginkel *et al.*, 1983; Wijffels *et al.*, 1991); an alginate or carrageenan solution containing suspended cells is extruded drop wise into respectively a Ca^{2+} or K^+ solution, initiating gelation of drops (Figure 1a). These immobilization techniques have been developed to entrap plant, animal, yeast, or bacterial cells and scaling up of these techniques was done with the 'resonance-nozzle' (Figure 1b; Hulst *et al.*, 1985; Hunik and Tramper, 1993).

Cultivation of the immobilized cells was done in air-lift loop reactors. This reactor type was chosen because it has no mechanically moving parts which can damage the support. The gel beads can easily be kept in suspension due to the liquid flow in these reactors. Furthermore, there is a good oxygen transfer from the gas to liquid phase. The hydrodynamic behaviour, mass transfer and mixing in air-

lift loop reactors were characterized in the presence and absence of a solid third phase, for example alginate gel beads (Bakker *et al.*, 1993; Verlaan, 1987).

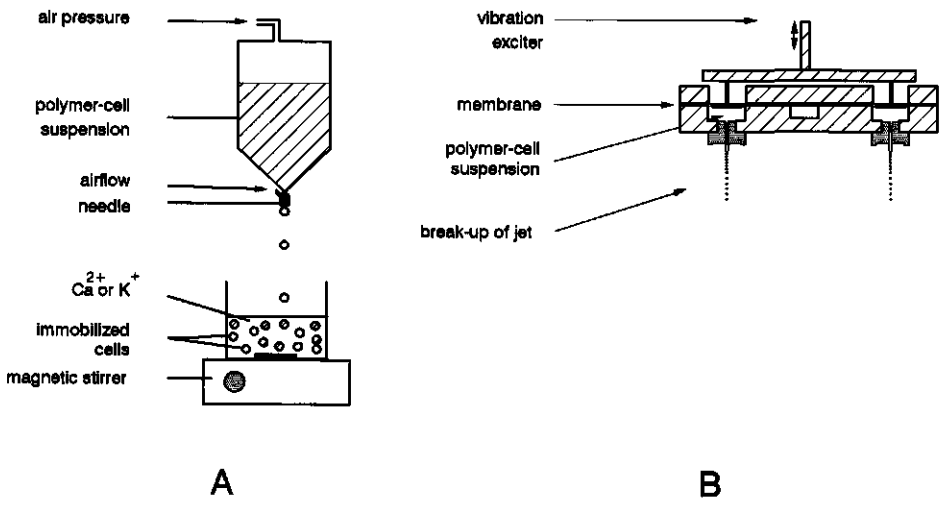

Figure 1. Schematic representation of entrapment techniques.
A) drop-wise extrusion;
B) the resonance nozzle with multiple jets.

The kinetics of immobilized cells were characterized and led to the development of dynamic models, which describe the substrate and biomass profiles in the support as well as the macroscopic oxygen consumption rate as a function of time. From the model results and from validating experiments it became evident which steps determine the rate of the overall process and to which parameters the immobilized-cell process is sensitive (De Gooijer *et al.*, 1991; Hunik *et al.*, 1994; Wijffels *et al.*, 1991). With this information it is possible to optimize the process and this forms the basis for optimal reactor design.

Furthermore, the model predictions and validating experiments showed that high capacities could be reached in such systems (Wijffels *et al.*, 1991). This has been the reason for considering the possibility of using immobilized cells not only as a model, but as a practical system as well.

In Figure 2 the approach, followed in our group, for the development of a process with immobilized cells is given.

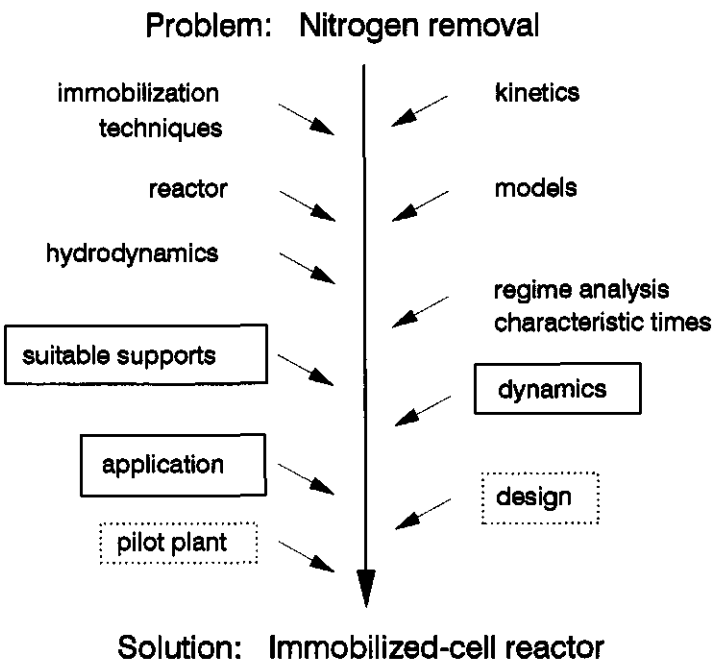

Figure 2. Schematic representation of the development of a process with immobilized cells (only text = previous research; ☐ studied in this thesis; ⋯ future research).

AIMS OF THIS THESIS

The main aim of the research described in this thesis was to obtain more insight in the complex processes occuring in artificially immobilized nitrifying cells. The knowledge obtained forms the basis for a rational design of compact systems for practical applications, e.g. nitrification of domestic wastewater. This knowledge was built up by developing dynamic models. In order to apply the immobilized-cell process for wastewater treatment the applicability and stability of the immobilization support must be investigated too. Therefore, support materials should be characterized and criteria to make a rational choice of the appropriate and optimal support material should be drawn.

DYNAMICS

In domestic-wastewater treatment, the process conditions are constantly changing. Treatment plants need to be designed in such a way that fluctuations have no significant negative effect on the effluent quality.

The dynamic models developed in our group are all based on general equations for kinetics of bacteria and transfer of substrate, meaning that they can be used in a general way. These models, however, describe the dynamics of a simplified immobilized-cell process from start-up until pseudo-steady state. They do not reflect the behaviour of the process as result of changes in for instance, temperature, pH, or substrate concentrations. In order to use these models for optimization of the immobilized-cell process and to come to a rational design for a compact nitrification reactor the effect of these fluctuations on the process have to be studied as well.

Model results can be used for optimal design if simulations are experimentally validated. This can be done at (1) reactor level by comparing simulated and observed macroscopic substrate consumption rates, based on the substrate concentrations in influent and effluent, and at (2) bead level by local measurements of biomass and substrate concentration profiles inside the support.

In dynamic systems the viability of the biomass is essential for the reaction of the system to fluctuations in process conditions. In Figure 3 the effect of biomass viability and death is demonstrated for 3 different situations (a, b, c). For instance, after an increase of temperature the overall consumption rate may increase too (a). At low temperatures, however, the substrate consumption rate of a cell will decrease. The substrate will then penetrate deeper into the support. If viable biomass is still present there, the substrate will be consumed deeper in the bead. The overall substrate consumption rate of the system will thus hardly drop (situation a). If, however, the cells in deeper layers are not viable anymore, less substrate will be consumed and the overall substrate consumption may drop substantially (situation b). If the cells have died due to the low temperature new biomass has to be produced to consume the substrate and therefore the system will react much slower to another change in temperature (situation c).
The same situations (a, b and c) are valid if the substrate load is increased or decreased.

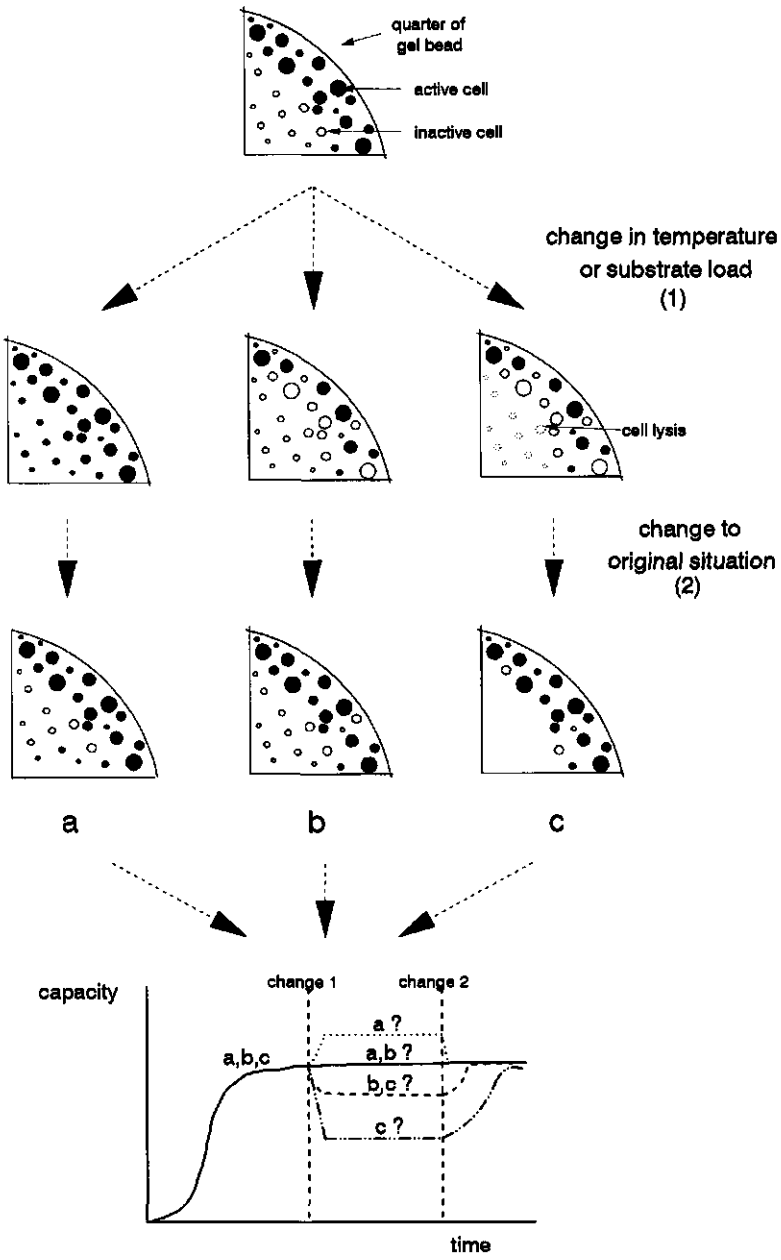

Figure 3. Schematic representation of the possible dynamics of immobilized cells.

Few methods have been developed for determination of biomass profiles in supports. Wijffels *et al.* (1991 and 1995) quantified the profiles by analyzing micrographs of median sections of carrageenan-gel beads by image analysis. Immobilized cells can also be stained with a β-emitting isotope, ^{32}S, supplied as sulphate and incorporated in proteins during synthesis. Growth can then be monitored as ^{32}S activity. Profiles can be observed with a microscope after exposure to an autoradiographic emulsion (Karel and Robertson, 1989).

Specific staining of m-RNA has shown to be applicable for monitoring specific degradations in wastewater and soil (Fleming *et al.*, 1993; Jeffrey *et al.*, 1994). A distinction between growing and non-growing cells can be made by staining RNA synthesis (Monbouquette *et al.*, 1990), or DNA synthesis (Boogert *et al.*, 1995; Kuhn *et al.*, 1991). A typical example of the last technique is shown in Figure 4. More research, however, is needed to obtain reliable quantitative results.

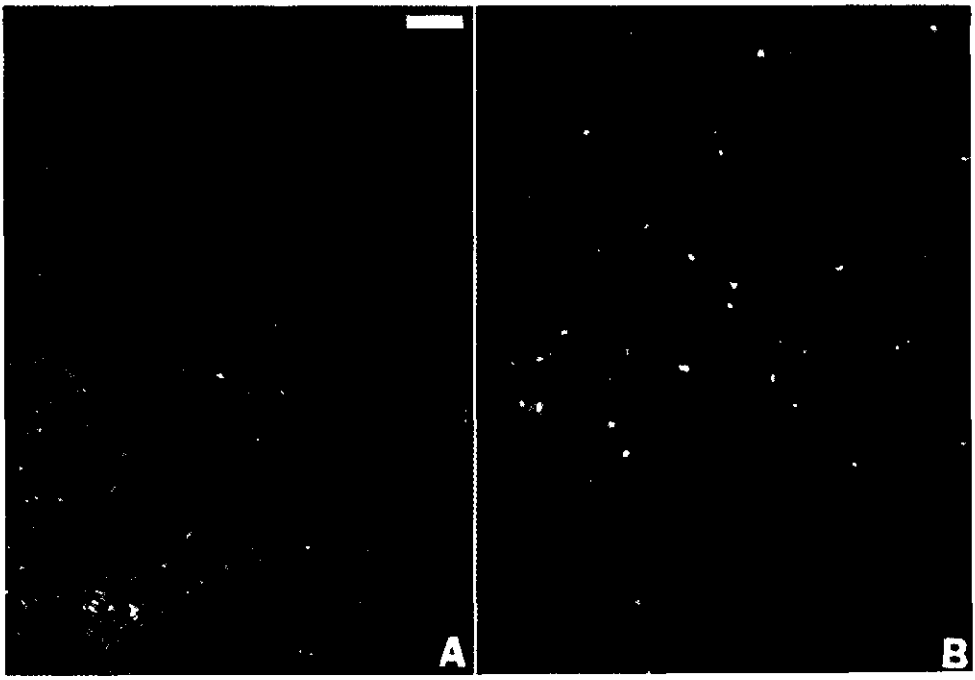

Figure 4. Discrimination between immobilized *Nitrosomonas europaea* cells with and without newly synthesized DNA. A) All DNA is stained; B) Only replicating cells which have incorporated bromodeoxyuredine (BrdU; thymine analogue) are stained (bar length 5 μm).

SUPPORTS

The life-time of the support is a crucial factor for the economic feasibility of the process. If the process with immobilized nitrifying cells is compared with activated-sludge treatment of domestic wastewater, the costs for production of alginate beads are compensated by the reduced costs for construction of a smaller reactor if the support remains stable for at least 2 months (Witteveen & Bos, 1991). Most of our previous research was done with beads made of natural polymers, such as *κ*-carrageenan and alginate. It is shown, however, that these materials are not suitable for application in wastewater, because they are biodegradable (Østgaard *et al.*, 1993[a] and 1993[b]), dissolve in domestic wastewater within a few days, and suffer from abrasion at high shear rates (Figure 5; Hunik, 1993). The alginate and carrageenan beads dissolve, because the stabilizing counter-ions (Ca^{2+} and K^{+}, respectively; Smidsrød and Skjåk-Bræk, 1990) are not sufficiently present in domestic wastewater. Therefore, a more stable support material has to be found.

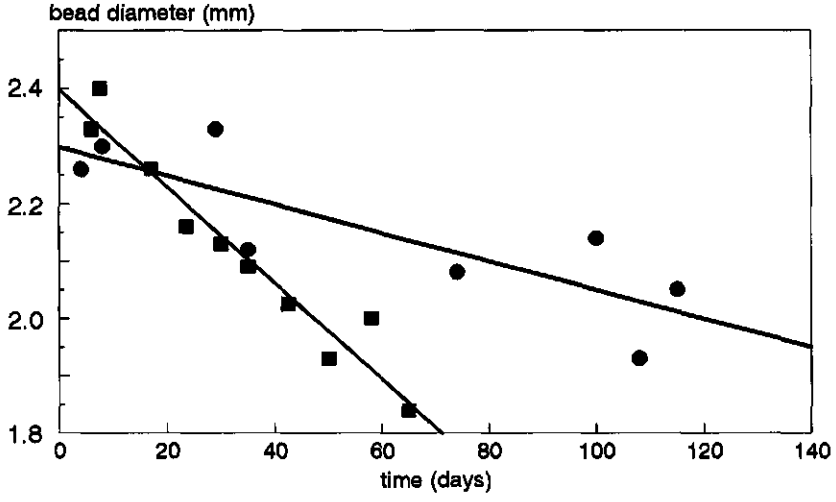

Figure 5. The diameter of carrageenan-gel beads as a function of time in reactors. Air-lift reactor of (●) 4 dm^3 and (■) 165 dm^3 (adapted from Hunik, 1993).

In order to establish a standard procedure for the assessment and comparison of the stability of supports, the mechanism of abrasion has to be disentangled and the relevant rheological characteristics have to be determined.

APPLICATION

Model studies on immobilized nitrifying cells entrapped in carrageenan, showed that it is possible to reach high nitrification capacities (2-4 kg $N.m^{-3}.d^{-1}$) in such systems (Hunik *et al.*, 1993; Wijffels *et al.*, 1991). These studies were done in well-defined synthetic media. In order to come to a rational design of an immobilized-cell process for treatment of domestic wastewater, the effect of replacing the synthetic medium with domestic wastewater and its constituents should be known.

The composition of domestic wastewater is constantly changing. In general, it consists (besides ammonium-containing compounds) of organic matter, colloidal and suspended solids, and heterotrophic organisms. In such media it is, in principle, possible that heterotrophic organisms attach to the support.

OUTLINE OF THIS THESIS

The chapters in this thesis can be read as independent articles. They are arranged according to three themes. The first section (Chapters 2 and 3) concentrates on the dynamics of immobilized nitrifying cells entrapped in carrageenan. In Chapter 2 the effect of low temperatures on the overall capacity is described, while in Chapter 3 the influence of varying substrate concentrations and cell death is presented. The second section (Chapters 4 and 5) deals with the selection and characterization of supports (Chapter 4), with emphasis on the relevance of rheological properties for the mechanical stability of supports in bioreactors (Chapter 5). Application of this system is the topic of the last section (Chapters 6 and 7). In Chapter 6 nitrification experiments in (pretreated) domestic wastewater with nitrifying cells entrapped in stable supports are described. Finally, in Chapter 7 the possibilities, drawbacks and prospects of this system for treatment of domestic wastewater are discussed.

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CHAPTER 2

REDUCED TEMPERATURE SENSITIVITY OF IMMOBILIZED *Nitrobacter agilis* CELLS CAUSED BY DIFFUSION LIMITATION

ABSTRACT

A dynamic model describing substrate consumption and growth of immobilized biomass was extended to predict and study the effect of variations in temperature. This was done by including temperature relations for all the individual parameters that are influenced by temperature. A sensitivity analysis was executed to identify the parameters that determine the temperature sensitivity of the overall process in the different cultivation phases. The model was evaluated by cultivating immobilized *Nitrobacter agilis* cells in an air-lift loop reactor at various temperatures between 6°C and 30°C. The dynamics of the system were studied by imposing temperature changes during the cultivation. The model describes the effect of low temperatures on the macroscopic consumption rate fairly well. As predicted, immobilized cells are less sensitive to temperature changes than suspended cells. The macroscopic consumption rate of immobilized *Nitrobacter agilis* cells at 10°C is around 70% of the macroscopic consumption rate at 30°C, while in the case of suspended cells this consumption rate is only 15%.

Reduced temperature sensitivity of *Nitrobacter agilis* cells caused by diffusion limitation.
E.J.T.M. Leenen, A.M.G.A. van Boxtel, G. Englund, J. Tramper and R.H. Wijffels.
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INTRODUCTION

The discharge of nitrogen compounds in the aquatic environment has become a severe problem for all aquatic areas, because they have a considerable stimulating effect on algal blooms (Zevenboom *et al.*, 1990). The biological removal of ammonia by nitrification and denitrification is widely used in wastewater treatment. In this process the nitrification rate is limited by the slow growth rate of the microorganisms involved.

Immobilization of nitrifying bacteria can be a successful strategy to improve biomass retention so that waste streams can be processed at high hydraulic rates. This can be achieved by spontaneous attachment to inert supports such as sand, lava or basalt (Gullicks and Cleasby, 1990; Murphy *et al.*, 1977; Tijhuis *et al.*, 1993), or by artificial entrapment in polymeric matrices (De Gooijer *et al.*, 1991; Lewandowski *et al.*, 1987; Tanaka *et al.*, 1991; Wijffels and Tramper, 1989; Wijffels *et al.*, 1991). In our research gel-immobilized nitrifying cultures are used.

The effect of temperature on immobilized cells was studied by Wijffels *et al.* (1995[b]): they showed that temperature effects on the activity of immobilized cells are masked when they are immobilized.

The present investigation concerns the performance of immobilized growing cells in the reactor. For this, the pseudo-homogeneous growth model of De Gooijer *et al.* (1991) is extended by implementation of temperature-dependent functions of the parameters. It is assumed that there is no diffusion limitation over the colonies, because the inoculum size is taken high enough to assure the formation of small colonies only (Wijffels *et al.*, 1994). A temperature sensitivity analysis is done with this extended model to identify the rate-determining parameter. The extended model is experimentally evaluated with immobilized *Nitrobacter agilis* in an air-lift loop reactor, especially considering the rate-determining parameters. In a first series of experiments cultivation is done at different temperatures, while in the second series of experiments cultivation is first done at optimal temperature and after some time the temperature is lowered to study the dynamics of the process.

MODEL DESCRIPTION

Description of dynamic processes should preferably be done with a dynamic model, which can predict not only steady-state situations but also the reaction of the process to changes. The dynamic model, used in this study, includes cell growth and substrate consumption in gel beads as a function of time. It predicts biomass and substrate concentration profiles across the bead and from that the overall substrate consumption by the immobilized cells.

External mass transfer is calculated by the film theory. Monod kinetics, in which maintenance requirements are taken into account, are used to calculate the macroscopic consumption rate of cells. Each time step a differential mass balance, over a bead in which simultaneous diffusion and consumption is occurring, is solved. For that, the gel bead is divided in 500 shells. The substrate consumption in each shell is calculated numerically using a second-order Runge-Kutta algorithm. After this the specific growth rate and the increase in biomass concentration in each shell is calculated (De Gooijer *et al.*, 1991; Wijffels *et al.*, 1991; Wijffels *et al.*, 1995[a]; see Appendix). The local biomass concentration was restricted to a maximum (11 $kg.m^{-3}_{gel}$); if more biomass is produced in the outer layers of the gel bead this biomass is excreted into the bulk liquid.

To predict the effect of variations in temperature the model was extended by including temperature relations for all the individual parameters which are influenced by temperature. The combination of these temperature effects results in the overall effect. Data from literature are used to obtain these relations. In our experimental set-up the bulk oxygen concentration was kept constant. Therefore, in this study, we do not consider the effect of temperature on the mass transfer from the gas phase and the bulk oxygen saturation constant. However, the effect of lowering the temperature would result in a higher oxygen concentration in the bulk phase. Including this effect in the model and experiments would result in a decreased temperature sensitivity of immobilized cells.

Effect of temperature

In the model all parameters which are influenced by temperature are adjusted with a temperature dependency. This is done by describing the effect of tempera-

ture with the Arrhenius equation. The Arrhenius equation is chosen because in this way it is possible to describe the temperature dependency of all parameters in a unified way. The Arrhenius equation is generally written as:

$$P = A \times e^{\left(\frac{-Ea}{R.T}\right)} \quad (1)$$

The Arrhenius constants and activation energies used in the model for the temperature sensitive parameters are given in Table I. If the activation energy is low the changes in temperatures have a limited effect on this parameter. A short discussion of the pertinent parameters is given below.

Table I. The Arrhenius values used in the model.

Parameter	Arrhenius constant	Activation Energy	Dimension
μ_{max}	1.167×10^{5}	58.4	s^{-1}
$D_{e,g}$	1.91×10^{-6}	17.2	$m^2.s^{-1}$
η_l	6.021×10^{-7}	18.1	Pa.s
K_s	1.57×10^{7}	52	$mole.m^{-3}$
m	3.915×10^{3}	38	$mole.kg^{-1}.s^{-1}$

Maximum specific growth rate

In literature several relations between the maximum specific growth rate (μ_{max}) and temperature have been described. They range from linear ones (Spencer and baines, 1964) to Arrhenius-related equations. Zwietering *et al.* (1991) investigated the different relations for the whole growth curve by statistical analysis of a large number of data points. In this research we are interested in the effect of low temperatures only. Therefore it is not necessary to describe the whole curve but only the part from the optimum to the minimum temperature. The Arrhenius equation describes such a curve well with only 2 parameters. Wijffels *et al.* (1995[b]) measured the maximum volumetric activity (r_s) of *Nitrobacter agilis* at temperatures varying from 2 to 32°C. As no information was available of the temperature sensitivity of the growth rate of nitrifying cells these data are used to calculate μ_{max} according to the following equation:

$$\mu_{max} = \frac{-Y_{xs} \times r_s}{X} \quad (2)$$

The biomass yield (Y_{xs} = 1.16x10^{-3} kg.mole^{-1}) is considered temperature independent, because of its stoichiometric nature (Heijnen and Roels, 1981). The biomass concentration (X) was 2.19x10^{-2} kg.m^{-3}. Maintenance requirements were neglected, because the maximal effect of the maintenance on this relation is calculated, according to Van 't Riet and Tramper (1991), to be 1% for *Nitrobacter agilis*.

The activation energy found (58.4 kJ.mole^{-1}) is comparable to the activation energies found by other authors (40-54 kJ.mole^{-1}; Helder and De Vries, 1983; Knowles *et al.*, 1965; Stratton and McCarthy, 1967).

Diffusion coefficient

The Arrhenius equation for the effective diffusion coefficient of oxygen in the gel beads ($D_{e,g}$) was determined by Wijffels *et al.* (1995[b]). The activation energy found for $D_{e,g}$ is considerably lower than the one found for μ_{max} (respectively 17.2 and 58.4 kJ.mole^{-1}); $D_{e,g}$ is thus less sensitive to temperature changes.

Liquid/solid mass transfer coefficient

In the equations for the calculation of the liquid/solid mass transfer coefficient ($k_{l,s}$; see Appendix) four parameters are temperature dependent, i.e. $D_{o,l}$ (as described by Wise and Houghton, 1966) the dynamic viscosity (η_l) of water (in the term Ga) and the densities of water (ρ_l in the Ga) and of the gel (ρ_p in the term Ga). The Arrhenius equation for η_l was derived by fitting this equation with a Marquardt algorithm through the data of Weast and Astle (1980). The effect of temperature changes on the densities are limited between 0 and 30°C and therefore these parameters are assumed to be constant and equal to the value at 20°C and atmospheric pressure (ρ_l = 998 kg.m^{-3} and ρ_p = 1008 kg.m^{-3}).

Substrate affinity constant

In literature little information about the effect of temperature on the affinity constant (K_s) for oxygen can be found. Boon and Laudelout (1962) measured the affinity constant of suspended cells of *Nitrobacter winogradskyi*. They found an activation energy of 52 kJ.mole^{-1} for temperatures between 20 and 35°C. This activation energy combined with the known K_s of *Nitrobacter agilis* at 30°C (0.017 mole.m^{-3}) results in the Arrhenius constant shown in Table I, using Eq. 1.

Maintenance and yield coefficient

Heijnen and Roels (1981) studied the effect of temperature on the maintenance coefficient m of a large number of aerobic microorganisms. Maintenance was considered as a first-order kinetics turnover process of macromolecules and therefore temperature dependency was expected. They calculated an activation energy of 38 kJ.mole^{-1}. With this value and the known maintenance coefficient for *Nitrobacter agilis* at 30 °C (1.1 x10^{-3} mole.kg^{-1}.s^{-1}) the Arrhenius constant is derived, using eq. 1 (Table I).

The biomass yield (Y_{xs} = 1.16x10^{-3} kg.mole^{-1}) is considered temperature independent, because of its assumed stoichiometric nature (Heijnen and Roels, 1981).

MATERIALS AND METHODS

Microorganism

Nitrobacter agilis (ATCC 14123) was cultivated in a 3 dm^3 chemostat at a dilution rate of 3.5x10^{-6} s^{-1} at 30°C. The medium contained per m^3 of demineralized water: 14.5 mole $NaNO_2$; 0.20 mole $MgSO_4$; 2.44 mole NaH_2PO_4; 7.56 mole Na_2HPO_4; 1 mmole Na_2MoO_4; 0.015 mmole $ZnSO_4$; 0.016 mmole $CuSO_4$ and 5 mmole $CaCl_2$. Per mole nitrite 0.02 mole $NaHCO_3$ was added. The pH of the medium was adjusted to 7.8 with 2 M KOH.

Immobilization procedure

Nitrobacter agilis cells, collected from the chemostat, were concentrated by centrifugation (10 min at 16,300g and 5°C) and washed with 15.4 mM NaCl. A 3% (w/v) κ-carrageenan solution (Genugel X-0909, A/S Kobenhavns Pektinfabrik, DK Lille Skensved) was gently mixed with the washed cell suspension, yielding a 2.6% (w/v) carrageenan solution.

Immobilization was done with the dripping technique or the resonance nozzle (Hulst *et al.*, 1985). The droplets were collected in 0.75 M KCl with a decane layer on top, to obtain perfect spheres (Buitelaar *et al.*, 1989). In order to initiate gelation the KCl solution and the decane were kept at 5°C.

Evaluation of the model results

Reduced temperature effects have been determined in six different experiments. The immobilized cells were cultivated in air-lift loop reactors at constant dilution rate. In four reactors (R1 - R4) the cultivation temperature was kept constant at respectively 30°, 15°, 11° and 6°C. To study the dynamics of the process temperature changes were applied in the reactor that was first operated at 30°C (R1). The temperature was lowered gradually from 30°C to 4°C. In addition model results were compared with previous published data of Wijffels *et al.* (1990;R5 - R6). In these experiments the temperature was lowered after 48 days from 30° to 20°C or 12°C, respectively.

The absolute bulk oxygen concentration in the reactors was kept constant (Table II) adapting for temperature by regularly regulating the flow of respectively air or oxygen manually (constant temperature) or with the aid of a programmable logic controller (PLC Melsec-G, Mitsubishi G626), which keeps the gasflow constant and mixes nitrogen and oxygen gas, as described by Wijffels *et al.* (1991). The medium composition was the same as for the chemostat, except for the nitrite concentration and the sodium salts. The latter were replaced by potassium salts and 10.8 mole.m^{-3} KCl was added to stabilize the carrageenan. The

influent nitrite concentration was adjusted a few times a week to keep the bulk nitrite concentration between 5 and 20 mole.m^{-3}. In this way oxygen was kept the limiting substrate while preventing toxicity problems caused by nitrite. The reactor specifications of the six experiments done are shown in Table II.

Table IIa. Specifications of the reactors. Cultivation at a constant temperature.

	R1	R2	R3	R4	R5	R6		
volume	3.2	3.2	2.9	3.2	3.0	3.25		[dm^3]
dilution rate	3.97	3.97	3.97	3.97	5.6	5.6	$x10^{-2}$	[s^{-1}]
gas flow rate	6.0	4.83	5.0	6.17	16.7	3.33	$x10^{-6}$	[$m^3.s^{-1}$]
bulk O_2	0.134	0.225	0.39	0.28	0.116	0.116		[mole.m^{-3}]
temperature	30	15	6	11	30	30		[°C]
initial biomass	6.5	6.5	6.5	11.3	45	45	$x10^{-4}$	[kg.m^{-3}]
bead radius	0.95	0.95	0.95	0.95	1.05	1.05	$x10^{-3}$	[m]
gel load	10	10	10	10	15	15		[%]

Table IIb. Specifications of the reactors: Cultivation at changing temperatures.

	R1	R5	R6		
volume	3.2	3.0	3.25		[dm^3]
dilution rate	3.97	5.6	5.6	$x10^{-2}$	[s^{-1}]
gas flow rate	6.0	16.7	3.33	$x10^{-6}$	[$m^3.s^{-1}$]
start bulk O_2	0.134	0.116	0.116		[mole.m^{-3}]
start temperature	30	30	30		[°C]
temperature change	20, 16, 12, 8, 4, 30	20	12		[°C]
bulk O_2 change	0.225, 0.238, 0.247, 0.303, 0.378, 0.144				
initial biomass	6.5	45	45	$x10^{-4}$	[kg.m^{-3}]
bead radius	0.95	1.05	1.05	$x10^{-3}$	[m]
gel load	10	15	15		[%]

Oxygen consumption rate

Nitrobacter agilis oxidizes nitrite using a stoichiometric amount of oxygen. To estimate the overall oxygen consumption rate the nitrite consumption rate per amount of gel was determined regularly.

Nitrite and nitrate concentrations in influent and effluent were determined spectrophotometrically using an auto-analysis system (Skalar 5100; Greenberg *et al.*, 1985). For nitrate analysis the nitrate was first reduced to nitrite by passing the sample through a cadmium column.

RESULTS AND DISCUSSION

Temperature sensitivity analysis

A sensitivity analysis for the main parameters is executed with the model to identify the parameters that determine the temperature sensitivity of the process to the largest extent. Model predictions in which all parameters are made temperature sensitive at temperatures ranging from 5° to 30°C, are compared with predictions in which only one parameter is temperature dependent. Macroscopic consumption rates were calculated at temperatures ranging from 5°C to 30°C. The oxygen concentration in the bulk phase is kept constant in the calculations (0.116 mole.m^{-3}) as was done in the experiments (for each temperature). The analysis is executed in the dynamic phase (at day 15) and in steady state.

In Figure 1 it is shown that in the temperature sensitivity of the overall process in the dynamic phase is mainly determined by the temperature sensitivity of the maximum specific growth rate as the lines have almost the same course. The process is relatively insensitive to temperature effects for the diffusion coefficient ($D_{e,g}$) and the liquid/solid mass transfer coefficient ($k_{l,s}$).

In the steady state the system is rather insensitive to temperature changes. None of the parameters have important effects on the sensitivityof the overall process to temperature.

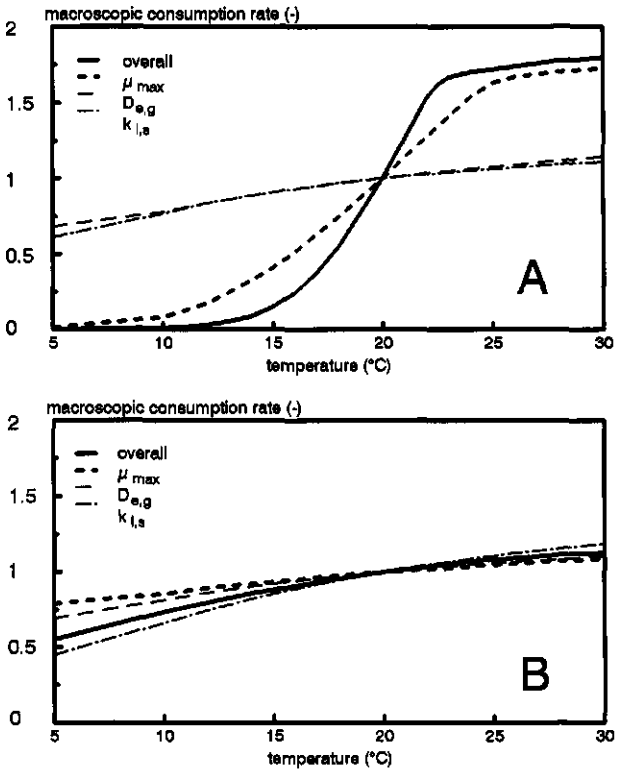

Figure 1. The oxygen consumption rate as a function of the temperature. Temperature sensitivity analysis for: A) the dynamic phase and B) the steady state.

Cultivation at different temperatures

Immobilized *Nitrobacter agilis* cells were cultivated in air-lift loop reactors at respectively 30°, 15°, 11° and 6°C. The oxygen consumption rates that were reached in the different experiments were compared with model predictions to evaluate the model (Figure 2).

At 30°C the maximum oxygen consumption rate was reached after 10 days of cultivation. The model describes the start-up of this experiment rather well, but the macroscopic consumption rate eventually reached was slightly lower than predicted. At lower temperatures this was the other way around; the start-up phase is not described satisfactorily while the reached maximum capacities are in better agreement with the model predictions. At 15°C the maximum consumption

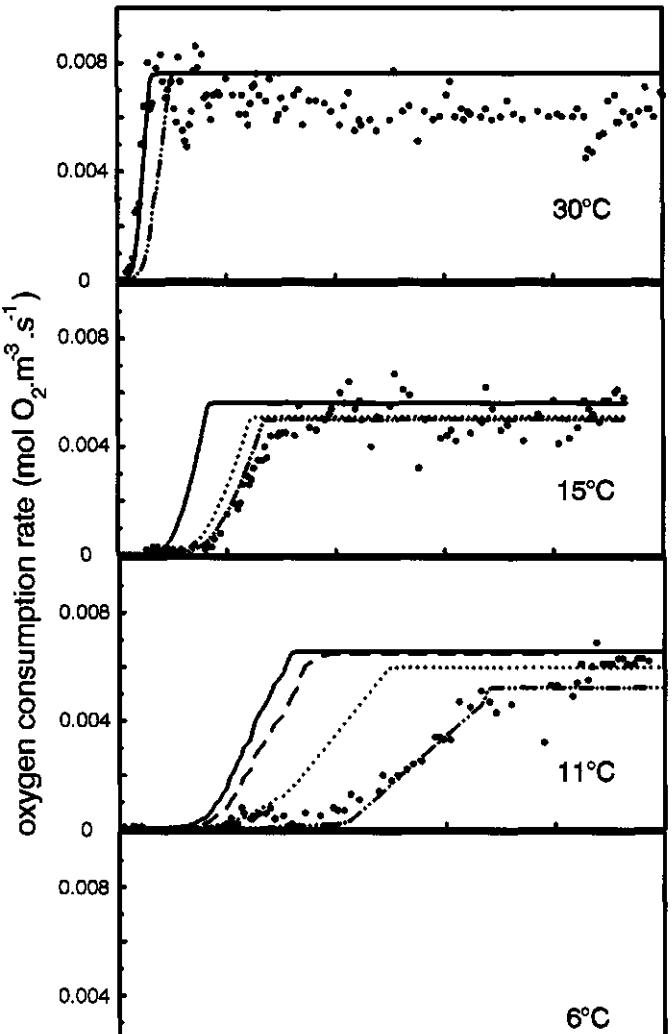

Figure 2. Experimental (●) and model results (————) of the macroscopic oxygen consumption rates of immobilized *Nitrobacter agilis* cultivated at 6, 11, 15 and 30°C. (model results obtained if the growth rate is 20% (·····) or 40% (-·-·-) lower than was estimated with the temperature sensitivity of the growth rate; (- - - - -) model results if the initial biomass concentration is 2 times lower).

rate was reached after approximately 70 days while the model predicts a start-up phase of about 40 days. This effect was even more profound at 11°C where the actual start-up phase is approximately 230 days while 60 days are predicted. At 6°C both the model predictions and the experimental results showed hardly nitrification during 230 days.

The differences between the model and experimental results can be explained with the temperature sensitivity analysis (Figure 1). According to this analysis the growth rate of the nitrifying bacteria is the parameter that determines the temperature sensitivity of the overall process in the dynamic phase. Therefore the differences between model and experimental results in the dynamic phase are very likely to be explained by the growth rate of the biomass. The growth rate is strongly dependent on the temperature (Ea = 58.4 $kJ.mole^{-1}$). Estimations of the growth rate are based on the values of the maximum volumetric activity of *Nitrobacter agilis* obtained by Wijffels *et al.* (1995[b]). At 30°C the parameter value used gives satisfactory predictions. At lower temperatures, however, the predictions are less accurate. Inaccuracies in the parameter values have a high effect on the overall performance at these process conditions. The effect of the growth rate for the experiments done at 30°C, 15°C and 11°C is shown in Figure 2 too; if the growth rate decreases more then was estimated with the expected temperature dependency (20% or 40% lower then was calculated with Arrhenius) of the growth rate different model curves were derived. The obtained curves simulate the experimental results better at 15°C and 11°C. The discrepancy between the model simulations and the experimental results are thus very likely of the temperature sensitivity of the growth rate, which is rate determining in the dynamic phase. An additional reason for the discrepancy between model and experimental results may be the value of the initial biomass concentration, which is hard to determine accurately. It is unknown how many cells remain viable during immobilization. If an error were made by a factor 2 this would lead to a shift of the time that steady state is reached by 1 day at 30°C. At 11°C, however, this would result in a difference of more than 10 days (see Figure 2). At the moment staining techniques are being developed to discriminate between living and dead cells in immobilized systems (Leenen *et al.*, 1996), which eventually may be used to determine the amount of cells that remain viable after immobilization.

The macroscopic consumption rate reached is less sensitive to temperature. In this situation the model is hardly influenced by temperature changes, the relatively

temperature insensitive liquid/solid mass transfer is rate determining (Figure 1b). The insensitivity in this phase is shown both in these experiments and model predictions. The macroscopic oxygen consumption rates reached at 11° and 15°C are still more than 70% of the consumption rate at 30°C (Figure 2).

Dynamics

Immobilized *Nitrobacter agilis* was cultivated in two air-lift loop reactors at 30°C. To study the dynamics of this process the temperature in the reactors was lowered after 48 days to respectively 12° and 20°C, while the oxygen concentration was kept constant at 0.12 mole. m^{-3}. In another experiment the temperature was lowered after 215 days from 30°C to subsequently 20°C, 16°, 12°, 8°, 4°C and finally the temperature was set back to 30°C again. In this experiment the bulk oxygen concentration was controlled manually (R1; Table II) and therefore some fluctuations in the bulk oxygen concentrations occurred. The macroscopic oxygen consumption rates were determined and compared with the model results (Figures 3 and 4).

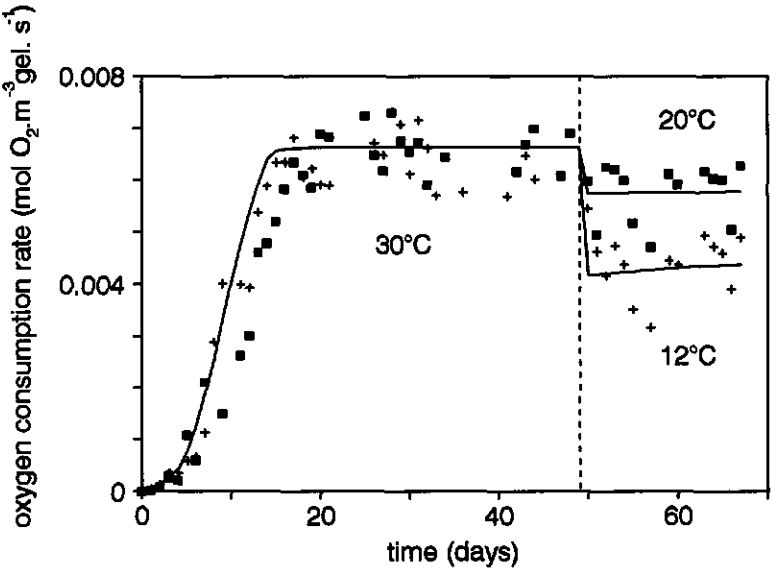

Figure 3. Experimental (+, ■) and model results (————) of 2 cultivations of immobilized *Nitrobacter agilis*. The first 48 days cultivation was at 30°C, after which the temperature was changed to 20 and 12°C, respectively.

The model describes the experimental results shown in Figure 3 very well, both in the start-up phase at 30°C and after lowering the temperature. A decrease in temperature from 30 to 20°C led to a decrease of 8-10% in the macroscopic oxygen consumption rate. A decrease in temperature from 30 to 12°C led to a decrease of 25-30%.

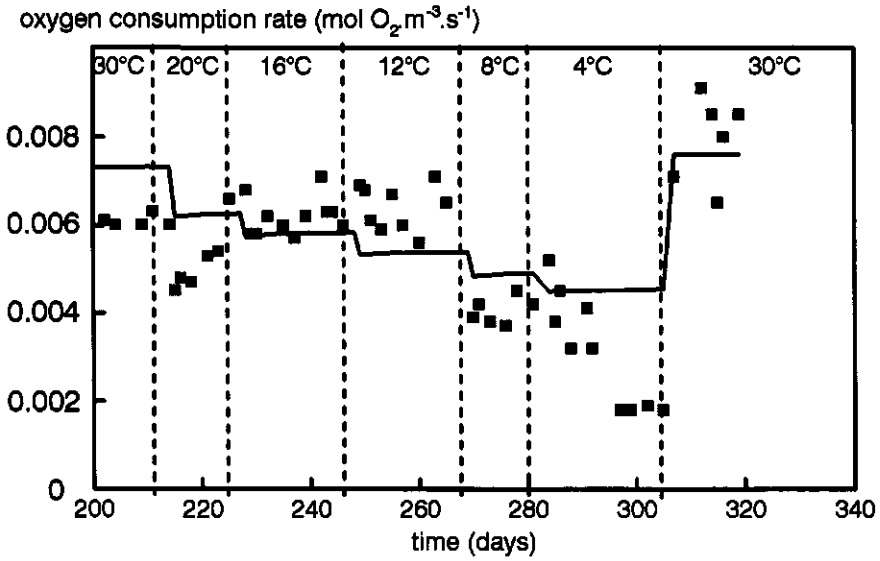

Figure 4. Experimental (■) and model results (————) of macroscopic oxygen consumption rate of immobilized *Nitrobacter agilis* cultivated at 30°C. After 215 days the temperature is changed steps wise via 20°C, 16°C, 12°C, 8°C to 4°C and back to 30°C again.

The experimental results shown in Figure 4 are less well described by the model. During the first temperature changes (until 12°C) the macroscopic oxygen consumption rate was almost constant. In addition to the low temperature sensitivity of the liquid/solid mass transfer coefficient ($k_{l,s}$; Figure 1) the bulk oxygen concentration increased at these temperature changes as well and therefore the nitrification rate stayed almost constant. The process seems to be more sensitive to temperatures below 8°C. At 4°C the model describes a constant oxygen consumption rate while in the experiment the rate decreases rapidly. After

changing the temperature back to 30°C the rate increases again to a somewhat higher level than was found before due to a slightly higher bulk oxygen concentration. The differences in the model predictions and experimental results can be explained by the differences in the bulk oxygen concentrations. During the experiment the bulk oxygen was tried to keep constant, but due to a higher bulk oxygen saturation constant at lower temperatures the bulk oxygen concentration was difficult to regulate. Therefore, sometimes it took a few days before a constant bulk oxygen concentration was reached. In the model simulations a constant bulk oxygen concentration, which was the mean value from the determined bulk oxygen concentration at a certain temperature (see Table IIb), was assumed.
From the results it becomes clear that the process adapts immediately to the new situation. Within a few days a new maximum consumption rate is reached. The macroscopic consumption rate in the reactor is reduced to a limited extent if the temperature is lowered. For this reason it would be much more convenient to start up the process at the optimum temperature and decrease temperature after the beads are grown full with biomass.

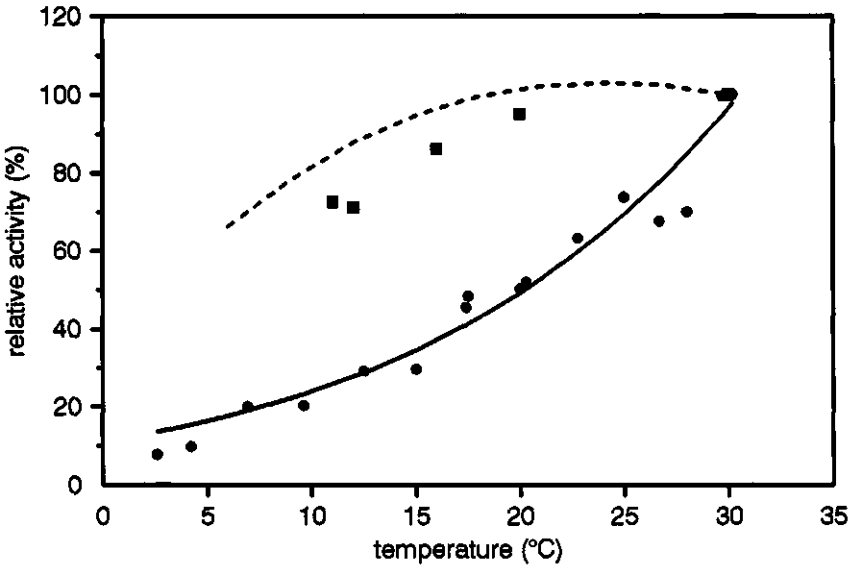

Figure 5. Maximum oxygen consumption of suspended (experimental results ●; model results ———) and immobilized *Nitrobacter agilis* (experimental values ■; model results – – –).

In Figure 5 simulated capacities reached at the whole temperature range are shown. In comparison the temperature effect on suspended cells, measured by Wijffels *et al.* (1995[b]), and the experimental values of the immobilized cells, found in this study, are given. This Figure shows that the model and experimental results are in good agreement. Also it is evident that immobilized cells are less sensitive to temperature changes than suspended cells. The temperature effects on the activity of immobilized are masked as a result of diffusion limitation. The relative capacities at lower temperatures are much higher than the relative capacities of the suspended cells. The macroscopic consumption rate of immobilized *Nitrobacter agilis* at 10°C is around 70% of the consumption rate at 30°C. In case of suspended cells this consumption rate is only 15%.

ACKNOWLEDGEMENT

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APPENDIX

Growth of biomass and consumption of substrate are treated separately, because the time scale for growth is several orders of magnitude larger than for substrate consumption (De Gooijer *et al.*, 1991).

The biomass production rate is defined as (Beeftink *et al.*, 1990):

$$r_x = \mu X - m Y_{xs} X\left(1 - \frac{S}{K_s + S}\right) \qquad (3)$$

The model is based on the consumption of one limiting substrate only. In our experimental set-up oxygen is always rate limiting. The rate of substrate utilization is given by:

$$r_s = \frac{\mu}{Y_{xs}} X + mX\left(\frac{S}{K_s + S}\right) \quad (4)$$

A differential mass balance over a biocatalyst bead in which simultaneous diffusion and consumption of substrate takes place was set up with the following assumptions:

- there is only a radial concentration gradient
- no convective transport occurs
- the biomass concentration is constant within one time step

The equation used in the integrating algorithm within one time step is:

$$D_e\left(\frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial S}{\partial r}\right)\right) = r_s + \frac{\partial S}{\partial t} \quad (5)$$

with the boundary conditions:

$$S = S_s \text{ at } r = r_b$$

$$\frac{\partial S}{\partial r} = 0 \quad at \quad r = 0 \quad or \quad r = r_f$$

To account for external mass transfer resistance, the film theory was used:

$$S_s^l = S_b - \frac{D_e}{k_{l,s}} \frac{\partial S}{\partial r}_{r=r_b} \quad (6)$$

The liquid/solid mass transfer coefficient ($k_{l,s}$) is calculated as follows:
For Reynolds (Re) > 30 the relation of Ranz and Marshall (1952) is used:

$$k_{l,s} = \frac{D_{o,l}(2 + 0.6\, Sc^{\frac{1}{3}} Re^{\frac{1}{2}})}{d_p} \quad (7)$$

For Re < 30 the $k_{l,s}$ is calculated with the relation of Brian and Hales (1969):

$$k_{l,s} = \frac{D_{o,l}}{d_p} \times \sqrt{4 + 1.21 \times (Re \times Sc)^{0.67}} \quad (8)$$

The Reynolds number was estimated by the Galileo number as was done by Wijffels *et al.* (1991).

NOMENCLATURE

A	=	Arrhenius constant (dimension depends on parameter)	
$D_{e,g}$	=	diffusion coefficient of oxygen in gel	$m^2.s^{-1}$
$D_{o,l}$	=	diffusion coefficient of oxygen in bulk	$m^2.s^{-1}$
d_p	=	diameter of gel particle	m
E_a	=	activation energy	$J.mole^{-1}$
g	=	gravitational acceleration (= 9.81)	$m.s^{-2}$
Ga	=	Galileo number	-
$k_{l,s}$	=	liquid/solid mass transfer coefficient	$m.s^{-1}$
K_s	=	substrate affinity constant for oxygen	$mole.m^{-3}$
m	=	maintenance coefficient	$mole.kg^{-1}.s^{-1}$
P	=	parameter value (dimension depends on parameter)	
R	=	gasconstant (= 8.314)	$J.mole^{-1}.K^{-1}$
Re	=	particle Reynolds number	-
r	=	distance from the centre of the bead	m
r_b	=	radius of the gel bead	m
r_f	=	distance from the centre of the bead where substrate concentration is negligible	m
r_s	=	volumetric activity	$mole.m^{-3}.s^{-1}$
r_x	=	biomass production rate	$kg.m^{-3}.s^{-1}$
S	=	substrate concentration	$mole.m^{-3}$
S_b	=	substrate concentration in bulk	$mole.m^{-3}$
S_s	=	substrate concentration at the surface of the bead	$mole.m^{-3}$
Sc	=	Schmidt number	-
T	=	temperature	K
t	=	time	s
X	=	biomass concentration	$kg.m^{-3}$
Y_{xs}	=	biomass yield	$kg.mole^{-1}$
η_l	=	dynamic viscosity of water	$kg.m^{-1}.s^{-1}$
ρ_l	=	density of water	$kg.m^{-3}$
ρ_p	=	density of gel particles	$kg.m^{-3}$

μ	=	specific growth rate	s^{-1}
μ_{max}	=	maximal specific growth rate	s^{-1}

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CHAPTER 3

DYNAMICS OF ARTIFICIALLY IMMOBILIZED *Nitrosomonas europaea*: Effect of biomass decay

ABSTRACT

The dynamics of growth and death of immobilized *Nitrosomonas europaea* were studied. For this, the death rate of suspended cells was determined in absence of ammonium or oxygen by following the loss of respiration activity and by fluorescein-diacetate (FDA)/lissamine-green staining techniques. The death rates obtained (1.06×10^{-6} s^{-1} or 4.97×10^{-6} s^{-1} in absence of oxygen or ammonium, respectively) were incorporated in a dynamic growth model and the effects on the performance of the immobilized-cell process illustrated by model simulations. These model simulations and experimental validation show that if decay of biomass occurs the biomass concentration in the centre of the bead decreases. As a result, the systems react slower to changes in substrate concentrations than if all cells remain viable.

In order to show that cells in the centre of the bead died, the FDA and lissamine-green staining techniques were adapted for immobilized cells. It was shown that biomass decay occurred, especially in the centre of the bead; the amount of cells decreased there and the remaining cells were all stained with lissamine green indicating cell death. After the substrate availability was decreased also cells near the surface of the bead lost their viability. The number of viable cells increased again after increasing the substrate concentration as the result of cell multiplication.

At low substrate concentrations and low hydraulic retention times, as for example in the treatment of domestic wastewater, the death rate of cells is thus an important parameter for the performance of the immobilized-cell system.

Dynamics of artificialy immobilized *Nitrosomonas europaea*: effect of biomass decay.
E.J.T.M. Leenen, A.A. Boogert, A.A.M. van Lammeren, J. Tramper and R.H. Wijffels.
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INTRODUCTION

Coupling of the biological processes of nitrification and denitrification is widely used for the removal of nitrogen from wastewater (Dos Santos *et al.*, 1996; Kuenen and Robertson, 1994). In such processes, however, the removal rate is limited by the slow growth rate of the nitrifying bacteria involved (Barnes and Bliss, 1983; Keen and Prosser, 1987). Immobilization of these bacteria can be a successful strategy to improve biomass retention, so that waste streams can be processed at high dilution rates (Hunik *et al.*, 1994; Wijffels *et al.*, 1991). In wastewater-treatment systems, however, the process conditions are continuously changing. Treatment systems need to be designed in such a way that fluctuations in for example water temperature and substrate loadings have no significant negative effect on the effluent quality.

Growth of immobilized cells has been studied extensively and dynamic models were developed describing the immobilized-cell process (De Gooijer *et al.*, 1991; Dos Santos *et al.*, 1996; Hunik *et al.*, 1994; Monbouquette *et al.*, 1990; Wijffels *et al.*, 1991 and 1995; Willaert, 1996). The effect of changes in temperature has been described previously (Leenen *et al.*, 1994 and 1996) and in the study presented here, the effect of biomass decay was investigated.

If the influent substrate concentration increases suddenly, the substrate penetration depth in the support material will increase as well. When cells in deeper layers are alive the immobilized-cell process will adapt instantaneously to this situation. If cells died due to the absence of substrate this adaptation will be slower. For prediction of the reactor response to changes in substrate concentration or volumetric load, the death rates must be considered.

A few methods have been developed for determination of biomass profiles in supports. Wijffels *et al.* (1991 and 1995) quantified the profiles by analyzing micrographs of median sections of carrageenan-gel beads by image analyses. Immobilized cells can also be detected with a β-emitting isotope, ^{32}S, supplied as sulphate and incorporated in proteins during synthesis. Growth can than be monitored as ^{32}S activity. Profiles can be observed with a microscope after exposure to an autoradiographic emulsion (Karel and Robertson, 1989). Specific staining of m-RNA has shown to be applicable for monitoring specific degradations in wastewater and soil (Fleming *et al.*, 1993; Jefrey *et al.*, 194). A distinction between growing and non-growing cells can be made by staining RNA

(Monbouquette *et al.*, 1990), or DNA synthesis (Kuhn *et al.*, 1991). The above-mentioned techniques provide information about spatial variation in colony distribution, growth or specific substrate degradation, but no information about the exact location of dead and viable cells inside gel beads. Therefore, staining techniques which discriminate between viable cells (FDA) and dead cells (lissamine green) are used in this study, both for the determination of the decay rate in cell suspensions and for detection of viable and dead immobilized cells.

The death rates obtained were incorporated in the model and the effect of biomass decay on the performance of the immobilized-cell process was illustrated by model simulations. Furthermore, the model was experimentally validated in an air-lift loop reactor with *Nitrosomonas europaea* immobilized in carrageenan. For this, the fluorescein-diacetate and lissamine-green staining techniques were adapted for immobilized cells. The main aim of this experiment was to show the importance of biomass decay for the dynamics of an immobilized-cell system.

THEORY

Model description

The dynamic model, evaluated in this study, is based on the model of Hunik *et al.* (1994), except that *Nitrosomonas europaea* is the only microbial species present in the gel bead. In this model the gel bead is divided in 250 equally spaced spherical shells and uniform conditions within each shell are assumed. The effective diffusion coefficient in the gel bead is assumed to be constant. The limiting substrate for *Nitrosomonas europaea* can be either ammonia or oxygen. Within each shell in the gel bead the most limiting substrate is determined according to the following Monod terms:

$$\frac{[O_2]}{K_{O_2} + [O_2]} \quad \text{and} \quad \frac{[NH_4^+]}{K_{NH_4^+} + [NH_4^+]} \qquad (1)$$

The most-limiting substrate (the substrate with the lowest Monod term) was used for calculations of substrate utilization. It is assumed that kinetics of the immobilized cells are similar to those of suspended cells.

The rate of substrate utilization is defined as (Beeftink *et al.*, 1990):

$$r_s = \frac{\mu}{Y_{xs}} X + mX\left(\frac{S}{K_S + S}\right) \quad (2)$$

The conversion of the non-limiting substrate is derived by the stoichiometry of the reaction equations.

The biomass is assumed to be homogeneously distributed at the start of the experiment and there is no transport of biomass within the gel bead. The biomass production rate for *Nitrosomonas europaea* is defined as (Beeftink *et al.*, 1990):

$$r_x = \mu X - mY_{xs}X\left(1 - \frac{S}{K_S + S}\right) \quad (3)$$

The biomass concentration is limited to a maximum (see Table III). When in the model the maximum biomass concentration in a shell is reached the excess biomass produced is assumed to be released into the bulk phase and washed out of the reactor (Hunik *et al.*, 1994). Growth of biomass and consumption of substrate are treated separately, because the time scale for growth is several orders of magnitude larger than for substrate consumption (De Gooijer *et al.*, 1991). The substrate concentration in the gel bead as a function of time and place is given by:

$$\frac{\partial S}{\partial t} = D_{gel}\left(\frac{\partial^2 S}{\partial r^2} + \frac{2}{r}.\frac{\partial S}{\partial r}\right) - r_s \quad (4)$$

with boundary conditions:

$$for \quad r = 0 \qquad -D_{gel}\frac{\partial S}{\partial r} = 0$$

$$for \quad r = R \qquad -D_{gel}\frac{\partial S}{\partial r} = k_l A\,(S_{bulk} - S_{sur})$$

The model is based on a completely mixed reactor and the mass balance of the substrate is thus given by:

$$\frac{dS_{bulk}}{dt} = D(S_{in} - S_{bulk}) - k_l A(S_{bulk} - S_{sur}) \quad (5)$$

where k_l is estimated according to the method described by Wijffels *et al.* (1991).

The ordinary differential equation (3) for the biomass growth in the gel beads was solved numerically with a second-order Runge-Kutta algorithm. The mass balance of the substrate (eq. 5) could be solved with an algorithm based on the trapezium rule. The substrate concentration profiles in the gel beads were calculated using a backwards-in-time and centered-in-space algorithm (BTCS) for partial differential equations. This finite-differences method allows changes in limiting substrate at any place in the gel bead and is in general conditions unconditional 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 (Hunik *et al.*, 1994).

Biomass decay

In our dynamic models (De Gooijer *et al.*, 1991; Hunik *et a.*, 1994) the growth model suggested by Beeftink *et al.* (1990), a combination of the models of Herbert (1959) and Pirt (1965), was used. The Herbert model defines growth-related consumption of substrate and maintenance requirements are derived from biomass decay, even in the presence of excess substrate. The Pirt model, on the other hand, assumes that substrate is used for growth and maintenance requirements, but negative growth (biomass decay) is not included. The Beeftink model (Eq. 3) integrates both approaches and views maintenance energy as being supplied from either substrate or biomass, depending on environmental conditions.

At very low substrate concentrations the model of Beeftink acts like the growth model of Herbert (1959):

$$r_s = \frac{\mu}{Y_{xs}} X \qquad (6)$$

$$r_x = (\mu - mY_{xs}) X \qquad (7)$$

If no substrate is present than $r_s = 0$ and $\mu = 0$. Equation 7 can than be rewritten:

$$r_x = -mY_{xs}X \quad \rightarrow \quad \ln\frac{X_t}{X_0} = -mY_{xs}t \qquad (8)$$

The decay rate of biomass ($k_d = mY_{xs}$) is therefore determined by the product of

the maintenance and the yield coefficient.

We determined the two decay rates in absence of oxygen or ammonium, and incorporated these values in the model as described by Hunik *et al.* (1994).

MATERIALS AND METHODS

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 growth 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 at 7.4 with 7 M $NaHCO_3$. The effluent of the chemostat 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 ca. 500 times concentrated cell suspension was used for testing the staining techniques, to determine the death rate and for immobilization.

Determination of the biomass decay rate

The cell suspension, obtained from 1.5 dm^3 effluent of the chemostat culture, was incubated batchwise in an aerated Erlenmeyer with 3 dm^3 growth medium with 75 mM NH_4Cl at 30°C. The pH was controlled at 7.5 with 7M $NaHCO_3$. To determine the biomass decay in the absence of oxygen, the ammonia and nitrite concentrations were determined in time, and as soon as 67% of the ammonia was consumed aeration was stopped and N_2 gas was sparged instead. Periodically a sample was taken and the maximal respiration activity of the cells (3 samples) was determined in a biological oxygen monitor, as a measure for the viable biomass concentration. The death rate was also measured in the absence of ammonium and the presence of oxygen. For this, as soon as the ammonium was consumed, the maximal respiration activity of the cells was followed as a function of time. Both set of experiments were done twice.

The ratio between the number of viable and dead cells was also determined with fluorescein-diacetate and lissamine-green staining by counting approximately 250 cells.

Respiration-activity assay

The viable biomass concentration was estimated by respiration-activity assays in a Biological Oxygen Monitor (BOM) as was described by Van Ginkel *et al.* (1983). With this BOM (Yellow Springs Instruments, Ohio, USA) the maximum oxygen consumption was measured. In an 8 cm^3 vessel a sample of the concentrated cell suspension was added together with 50 mM phosphate buffer (pH 7.5) to a volume of 4 cm^3 and aerated with oxygen for 10 min. Then the vessel was sealed with the oxygen electrode (model 5331, Yellow Springs Instruments, Ohio, USA) such that no air bubbles remained in the vessel. Concentrated $(NH_4)_2SO_4$ (1 M) was added through the seal with an analytical syringe (0.01 cm^3) to avoid a significant change in the liquid-volume. The decrease in oxygen concentration was then recorded as a function of time. These assays were done at 30°C. The respiration activity was defined as the initial oxygen consumption rate of the cells under the conditions applied in the vessel.

Staining techniques

Two staining techniques were used to discriminate between viable cells and cells with a degenerated cell membrane (here designated as dead). The fluorescein-diacetate labelling was used to label viable cells, and cells with degenerated membranes were labelled with lissamine green.

The fluorescein-diacetate and lissamine-green staining techniques were applied simultaneously to one sample. By changing from bright-field mode to UV the viable or dead cells could be counted in the same image.

Fluorescein diacetate (FDA)

The fluorescein-diacetate (FDA) labelling is based on the activity of esterases present in metabolic-active cells, which catalyse the reaction:
fluorescein diacetate (FDA) → fluorescein + 2 acetate
The colourless FDA is transported through an intact cell membrane. Inside viable cells esterases convert FDA to fluorescein. Fluorescein can not leave the cell through an intact cell membrane and is fluorescent under UV-excitation. The fluorescein will thus accumulate inside viable cells. Dead cells do not contain esterases and remain colourless (Chrzanowski *et al.*, 1984; Heslop-Harrison and Heslop-Harrison, 1970).

A stock-solution of 0.5% FDA (Sigma) in acetone was stored at -20°C. Just before use a 25 times dilution in growth medium was prepared.

Suspended cells were stained by adding one droplet of the FDA-solution to one droplet of cell suspension and the sample was observed with a fluorescence microscope (Nikon, Labophot) under UV-light (dichroic mirror DM 400, excitation filter Ex 365 and barrier filter BA 420).

Immobilized cells were stained by adding 0.5 ml FDA-solution to 10 gel beads. After approximately 5 minutes the gel beads were cut with a razor blade and sections were observed with a fluorescence microscope under UV-light. This procedure was selected after testing incubation times of 1, 5 or 30 minutes and addition of 0.2, 0.5 or 1.0 ml FDA-solution.

Lissamine green

Lissamine green is a selective stain for the cytoplasm of degenerating and degenerated cells, because it only permeates disrupted cell membranes. The lissamine green will accumulate as a result of the irreversible binding. It can not enter viable cells, thus no accumulation will occur there. Dead cells treated with lissamine green will, therefore, appear green under a microscope in bright-field mode (Van Lammeren, 1988).

A stock-solution of 0.2% lissamine green (Gurr) in medium was stored at -20°C.

Suspended cells were stained by adding one droplet of the lissamine-green solution to one droplet of cell suspension. After a few minutes the sample was observed with a microscope in bright-field mode. Viable cells were colourless and

dead cells were stained green.

Immobilized cells were stained by adding 0.5 ml lissamine-green solution to 10 gel beads. After approximately 5 minutes the gel beads were sectioned and observed with the microscope. Viable cells were colourless and dead cells were stained green. This procedure was selected after testing various incubation times (1,5 or 30 minutes) and lissamine-green quantities (0.2, 0.5 or 1.0 ml stock-solutions).

Immobilization procedure

A 3% (w/v) κ-carrageenan solution (Genugel X-0909, A/S Kobenhavns Pektinfabrik, DK Lille Skensved) was gently mixed with a concentrated suspension of washed cells, yielding a 2.6% (w/v) carrageenan solution.

Immobilization was done with the resonance nozzle (Hulst *et al.*, 1985). The droplets were collected in 0.75 M KCl with a decane layer on top, to obtain perfect spheres (Buitelaar *et al.*, 1989). In order to initiate gelation the KCl solution and the decane were kept at 5°C.

Macroscopic oxygen consumption rate

Nitrosomonas europaea oxidizes ammonia using a stoichiometric amount of oxygen (Barnes and Bliss, 1983; Keen and Prosser, 1987). To estimate the macroscopic oxygen consumption rate the ammonia consumption rate per amount of gel was determined regularly.

Ammonia and nitrite concentrations in influent and effluent were determined spectrophotometrically using an auto-analysis system (Skalar 5100; Greenberg *et al.*, 1985).

Evaluation of the model results

The immobilized cells were cultivated in an air-lift loop reactor (3.10^{-3} m^3) at 30°C and pH 7.4. The bulk oxygen concentration in the reactors was kept constant

(0.12 mole $O_2.m^{-3}$) by regulating the air flow with the aid of a programmable logic controller (PLC Melsec-G, Mitsubishi G626), which keeps the gas flow constant and mixes nitrogen and air, as described by Wijffels *et al.* (1991). The medium composition was the same as for the chemostat, except for the ammonium concentration and the sodium salts. The latter were replaced by potassium salts; 10.8 mole.m^{-3} KCl was added to stabilize the carrageenan. Approximately 10% of the reactor volume was occupied by carrageenan beads with entrapped *Nitrosomonas europaea*. To study the dynamics during the experiment the hydraulic retention time (HRT) and the influent ammonium concentration were varied. The exact conditions are given in Table I.

Table I. Influent ammonium concentrations, hydraulic retention times (HRT) and ammonium load during the experiment.

time (days)	influent NH_4Cl concentration (mM)	HRT (h)	Ammonium load (mole $NH_4Cl.m^{-3}.d^{-1}$)
0	4	1.25	77
10	5.25	1.25	101
11	4	1.25	77
14	3	0.7	103
28	6.5	1.6	96

Thickness of the biomass layer

During the experiment slides were made from (stained and not stained) sections of the gel beads with immobilized cells. These slides were used to determine the thickness of the biomass layer just beneath the surface of the bead. As long as the colonies were distributed homogeneously no layer was found (= 1000 μm = radius of gel bead). As soon as larger colonies were formed near the surface of the bead than in the deeper layers of the gel bead it was assumed that a biomass layer was formed. From that moment the thickness of the biomass layer was determined by determining where the colony radii were 40% of the radii in the large colonies near the surface. The mean value was derived by analyzing at least 15 different slides taken from sections of different gel beads.

RESULTS AND DISCUSSION

Determination of biomass decay

The decay rate of biomass was followed by determining the loss of respiration activity of suspended *Nitrosomonas europaea* in absence of oxygen or ammonium. The results are shown in Figure 1. The maximal respiration activity decreased in time, both under oxygen and ammonium depletion. From Figure 1 a decay rate of 1.06 x 10^{-6} s^{-1} (7% of μ_{max}) was calculated if oxygen was absent. The decay rate when ammonia was absent was found to be approximately 5 times higher (4.97 x 10^{-6} s^{-1}; 31% of μ_{max}). The reason for this difference in decay rate is not known yet; may be if ammonia is limiting oxygen radicals are formed (by the monooxygenases present) and reducing equivalents are needed to remove these (Laudelout *et al.*, 1968) which may increase the decay rate. From these results it becomes clear that if ammonia is the limiting substrate the oxygen concentration has to be decreased too to reduce biomass decay: *Nitrosomonas europaea* is more sensitive to ammonium than oxygen depletion.

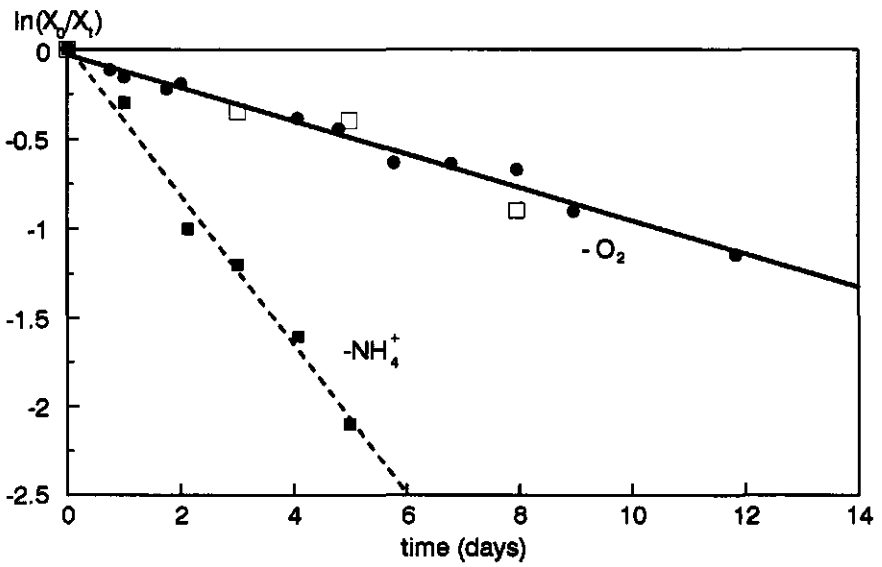

Figure 1. The death rate of a cell suspension of *Nitrosomonas europaea* in absence of either ammonium or oxygen (●, ■ data obtained by respiration-activity assay; □ data obtained with FDA/lissamine-green staining).

The information obtained from these experiments is whether or not cells can instantaneously recover their maximum respiration activity when substrate is added after a period of depletion. To confirm whether this loss of activity is an accurate measure, the ratio between viable and dead cells was determined using the combined FDA/lissamine-green staining techniques (Figure 1). FDA and lissamine-green stained cells were clearly distinguished in the same sample and all cells were stained. The cells which were colourless under UV were blue-green in bright field (dead cells). The cells which were fluorescent under UV were colourless in bright field (viable cells). The staining method was checked first with cells killed by incubating in 96% ethanol: no cells were fluorescent under UV and all cells were green in bright field. The FDA- and lissamine-green staining techniques were complementary and are thus useful to distinguish viable and dead cells in a suspended culture.

The results obtained with the respiration-activity assays are thus confirmed by the results obtained with the FDA/lissamine-green staining. The decrease in respiration activity is therefore a good measure for the biomass decay rate.
In literature only few authors presented data on the decay rate of pure strains of nitrifying cells (see Table II). The values found in this study correspond well with the data of all these authors and also with the values found for nitrifying sludge.

Table II: Decay rates of cells reported in literature

Organism	depletion	decay rate (s^{-1}) $x10^{-6}$	Reference
N. europaea	oxygen	1.36	Wijffels *et al.* (1995)
N.europaea + *N.agilis*	ammonia	1.2 - 6.4[#]	Laudelout *et al.* (1968)
N. europaea		2.88 - 7.35[$]	Keen and Prosser (1987)
nitrifying sludge		0.5 - 1.2	Barnes and Bliss (1983)
nitrifying sludge		2.31	Tanaka *et al.* (1996)

[#] dependent on the oxygen concentration
[$] maintenance and growth yield coefficients were determined. Decay = m x Y_{xs}.

Model simulations

The dynamic model, developed in this study, was used to illustrate the effect of biomass decay on the performance of the immobilized-cell process. All input parameters used in the dynamic model were obtained from the literature or determined in separate experiments previously done in our group (Table III). The determined decay rates were incorporated in the model. The simulations were done as if decay of biomass was absent ($k_d = 0$) and as if decay of biomass was occurring with the determined decay rates, depending on the rate-determining substrate. In the inner part of the gel bead another substrate can be limiting than in the outer parts of the gel bead, due to differences in diffusion coefficients and consumption rates.

Table III: Input parameters used in the dynamic model

parameter	value	dimension	source
μ_{max}	1.59×10^{-5}	s^{-1}	average of several literature values (Hunik *et al.*, 1994)
K_{NH4+}	1.25	$mole.m^{-3}$	Hunik *et al.* 1992
K_{O2}	5.05×10^{-3}	$mole.m^{-3}$	Hunik *et al.* 1992
$k_{d,NH4+}$	4.97×10^{-6}	s^{-1}	this article
$k_{d,O2}$	1.06×10^{-6}	s^{-1}	this article
X_{max}	11	$kg.(m\ gel)^{-3}$	Wijffels *et al.* 1991
X_0	6.5×10^{-4}	$kg.(m\ gel)^{-3}$	determined at start experiment
R	9.5×10^{-4}	m	determined at start experiment
$k_{l,NH4+}$	2.65×10^{-5}	$m.s^{-1}$	calculated as described by Wijffels *et al.* 1991
$k_{l,O2}$	3.13×10^{-5}	$m.s^{-1}$	calculated as described by Wijffels *et al.* 1991
$D_{gel,NH4+}$	1.9×10^{-9}	$m^2.s^{-1}$	average of several literature values (Hunik *et al.*, 1994)
$D_{gel,O2}$	2.05×10^{-9}	$m^2.s^{-1}$	Wijffels *et al.* 1994

The effect of biomass decay on the development of the biomass profile across the gel bead is simulated (Figure 2). In Figure 2 is shown that during cultivation a biomass profile develops. The cells near the surface of the bead (relative radius = 1) have a high growth rate, because the substrate concentration is high. The cells in the core of the bead (relative radius = 0) have less substrate and therefore their growth rate is lower. If biomass grows according to the model with the incorporated death rates the increase in biomass concentration is slower than when no cell death occurs.

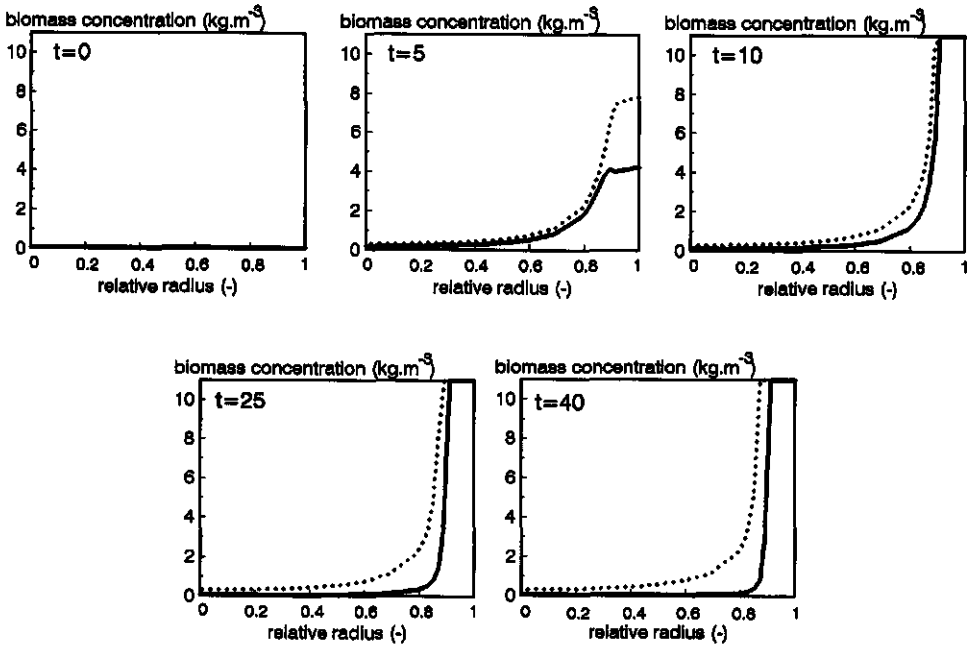

Figure 2. Model predictions of the development of a biomass profile in a gel bead with (———) and without (----) the occurrence of biomass decay cultivated for 0 - 40 days. The simulations are done with an influent ammonium concentration of 5 mM, a bulk oxygen concentration of 0.12 mM oxygen and a hydraulic retention time of 1 hour (0 = centre of bead; 1 = edge of the bead).

After a few days hardly any substrate will reach the core of the bead, because the cells at the outer parts of the beads will consume all substrate. Due to this, no biomass growth will occur any longer in the core. If no biomass death occurs the cells in the centre of the bead will remain viable, while when cells die the biomass concentration will slowly decrease. This effects the dynamics of the overall system. If, for example, all biomass is still present when the bulk substrate concentration is increased suddenly (substrate diffuses into deeper layers of the gel bead), the influent substrate will be consumed immediately by the biomass present in the deeper layers. If less viable biomass is present due to cell death, cells that remained alive have to replicate again to be able to react to this change in substrate concentration. In these model simulations, the biomass concentrations reached after 40 days of cultivation were respectively 84 $kg.m^{-3}$ (without biomass decay) and 50 $kg.m^{-3}$ (with biomass decay), while the overall macroscopic conversion rates were approximately the same, respectively 0.0065 and 0.0063 mole $O_2.m^{-3}.s^{-1}$.

The effect of biomass decay on the effluent quality is also simulated (Figure 3). In this simulation the bulk oxygen concentration (0.12 mM $\approx$ 50% saturation) and the influent ammonium concentration (3 mM) were constant. The hydraulic retention time (HRT) was changed after 20 days from 1 to 5 h and after 70 days back to 1 h again.

In the first period the growth rate of the biomass is lower if decay of biomass is occurring (decay = maintenance x yield coefficient); thus the effluent ammonium concentration decreases slower and reaches a higher level than if no biomass decay occurred. After a decrease in load (increase in HRT) the biomass will get less ammonium. When all cells remain viable ammonium will be consumed almost completely. When, however, cells die this will result in a decrease in the biomass concentration. The effluent ammonium concentration will slowly increase until pseudo-steady state. In this case the conversion will be lower. When all cells remain viable the conversion will, in this model simulation, be about 95%, while if biomass decay is occurring the conversion is about 80%. When the substrate load increases again (HRT decreases) the system will react instantaneously to the new situation if all cells are still viable. In the other case, when cell death has occurred, not enough viable biomass will be present any more to convert the ammonium. During approximately 7 days new biomass has to be produced before pseudo-steady state is reached again.

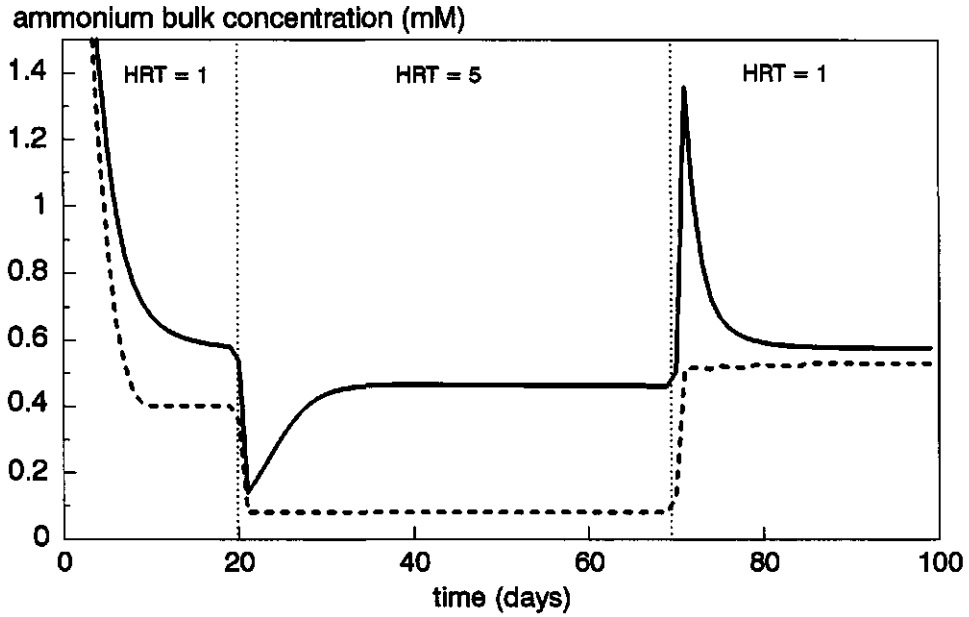

Figure 3. Model predictions of the effluent ammonium concentrations at changing hydraulic retention times (HRT; ammonium load) with (———) or without (-----) the occurrence of biomass decay. The simulations are done with an influent ammonium concentration of 3 mM and a bulk oxygen concentration of 0.12 mM. At t=0 days the HRT=1 h, at t=20 days the HRT=5 h and at t=70 days the HRT=1 h again.

These simulations clearly show that biomass decay is an important parameter for the dynamics of the immobilized-cell system.

Applicability of the staining techniques for immobilized cells

The FDA and lissamine-green staining procedures used for suspended cells were also used for immobilized cells. We expected that adaptations might be necessary as reagents need to diffuse to the cells. Therefore, different incubation times and amounts of FDA or lissamine-green solutions were investigated.

All combinations tested (incubation times of 1, 5 and 30 minutes and FDA or lissamine-green solutions of 0.2, 0.5 or 1.0 ml) gave approximately the same results; all cells were stained with FDA or lissamine green. Therefore, we concluded that FDA and lissamine green diffuse into the matrix easily. In the further research an incubation time of 5 minutes with 0.5 ml FDA or lissamine-green solution was used.

Reactor performance

Nitrosomonas europaea cells immobilized in carrageenan were cultivated in an air-lift loop reactor. To study the effect of growth and death on the dynamics of biomass during the experiment the hydraulic retention time (HRT) and the influent ammonium concentration were varied (Table I). During this experiment the overall reactor performance was followed by analyzing the substrate and product concentrations. One experiment was done, because previous studies (Hunik *et al.*, 1994; Wijffels *et al.*, 1991 and 1995) have shown the reproducibility of these kind of experiments. The main aim of this experiment was to show that biomass decay may be important for the dynamics of immobilized cells.

In Figure 4 the experimentally determined nitrite and ammonium concentrations are compared with the model simulations. The model simulations are done under two different conditions: with and without decay of biomass. Until day 11 the model predictions and experimental data deviate. These deviations are probably due to an overestimation of the amount of viable cells at the start of the experiment and some practical problems, which resulted in an increase in ammonium concentration. After 11 days the model predictions and experimental data correspond well. After 14 days the hydraulic retention time and the ammonium concentration were decreased. The immobilized-cell system responds instantaneously and the obtained nitrite concentrations are well predicted. The simulations of the model in which biomass decay is taken into account correspond slightly better with the experimentally obtained data than the simulations done when all cells remain viable. This difference is most profound after 28 days when the HRT and the ammonium concentration were increased again.

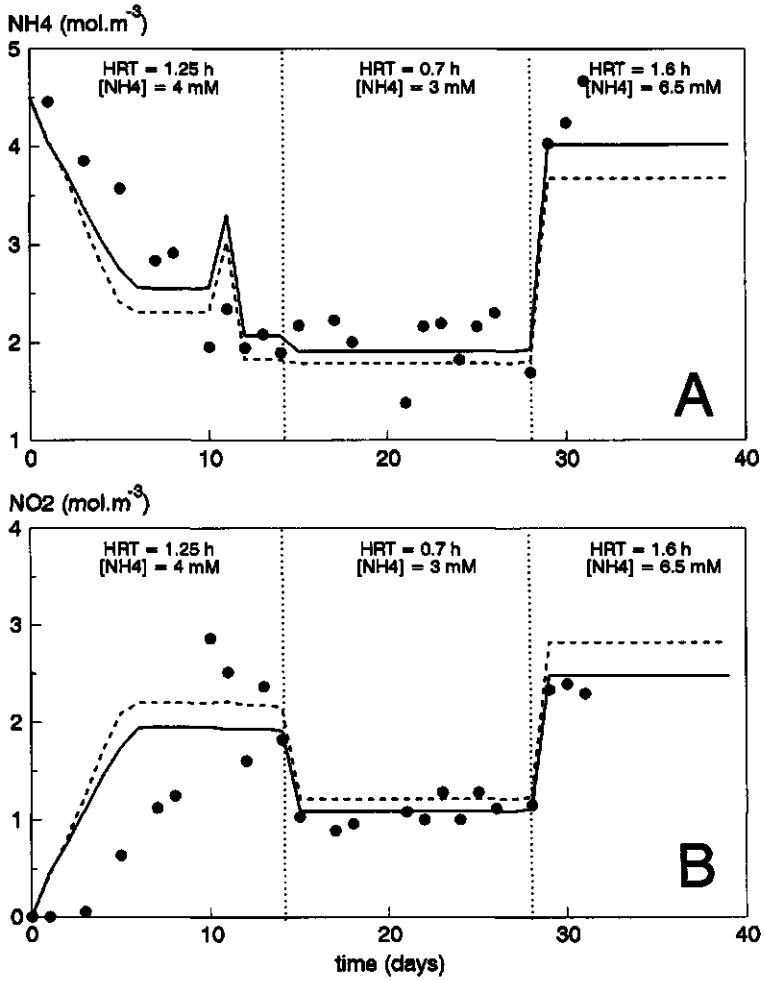

Figure 4. Effluent ammonium and nitrite concentrations (●) in comparison with model predictions (——— with biomass decay; —— without biomass decay).
A) Effluent ammonium concentrations;
B) Effluent nitrite concentrations.

From these results it is concluded that the results of the model in which the separately determined decay rates are incorporated match slightly better with the experimental data.

Growth dynamics

The viability and development of a biomass profile of immobilized *Nitrosomonas europaea* were followed microscopically during the experiment with the aid of the FDA and lissamine-green staining techniques. Typical images of sections of gel beads with immobilized *Nitrosomonas europaea* are shown in Figure 5A; Figure 5B is a schematic representation of these images. Dead cells are dark-green in bright field (I) and not visible in UV (II), viable cells are colourless in bright field (I) and fluorescent in UV (II). During the first 4 days the biomass was homogeneously distributed in the gel bead and all cells were viable. From approximately day 7 on a biomass profile became evident. In an outer layer of approximately 125 μm colonies with a diameter of about 11 μm developed, while in the centre of the bead the colonies were clearly smaller; all biomass was still viable. The next days the biomass profile became steeper. After 10 days 90% of the detected biomass was still viable and large colonies with a diameter around 25 μm had developed near the surface of the bead. In these colonies both dead and viable cells were detected by showing both fluorescence in UV and a few green cells in bright field. After 13 days large colonies (diameter $\approx$ 30 μm) were present at the edge of the bead; in a layer of approximately 80 μm colonies with diameters larger than 10 μm were present and in deeper layers smaller colonies were observed.

At day 14 the ammonium concentration and the HRT were decreased. After this change about 60% of the detected biomass appeared to be viable; this viability was only found near the surface of the bead. The biomass in the centre of the bead was not able to convert FDA and all had accumulated lissamine green; all these cells were thus dead. After 17 days even less cells (approximately 25%) were found to be viable. Also cells near the surface of the bead lost there viability. The next few days more biomass death and decay were observed and after 22 days only about 10-15% of the observed cells emitted light under UV (metabolic-active cells).

At day 28 the HRT and ammonium concentration were increased. Figure 6 clearly shows that after 30 days more viable cells were present again near the surface of the bead (80% of the observed biomass). Hardly any biomass at all is present any more in the centre of the gel bead. Clearly biomass decay had occurred there and almost all cells were dead; only a few colonies with dead cells were detected.

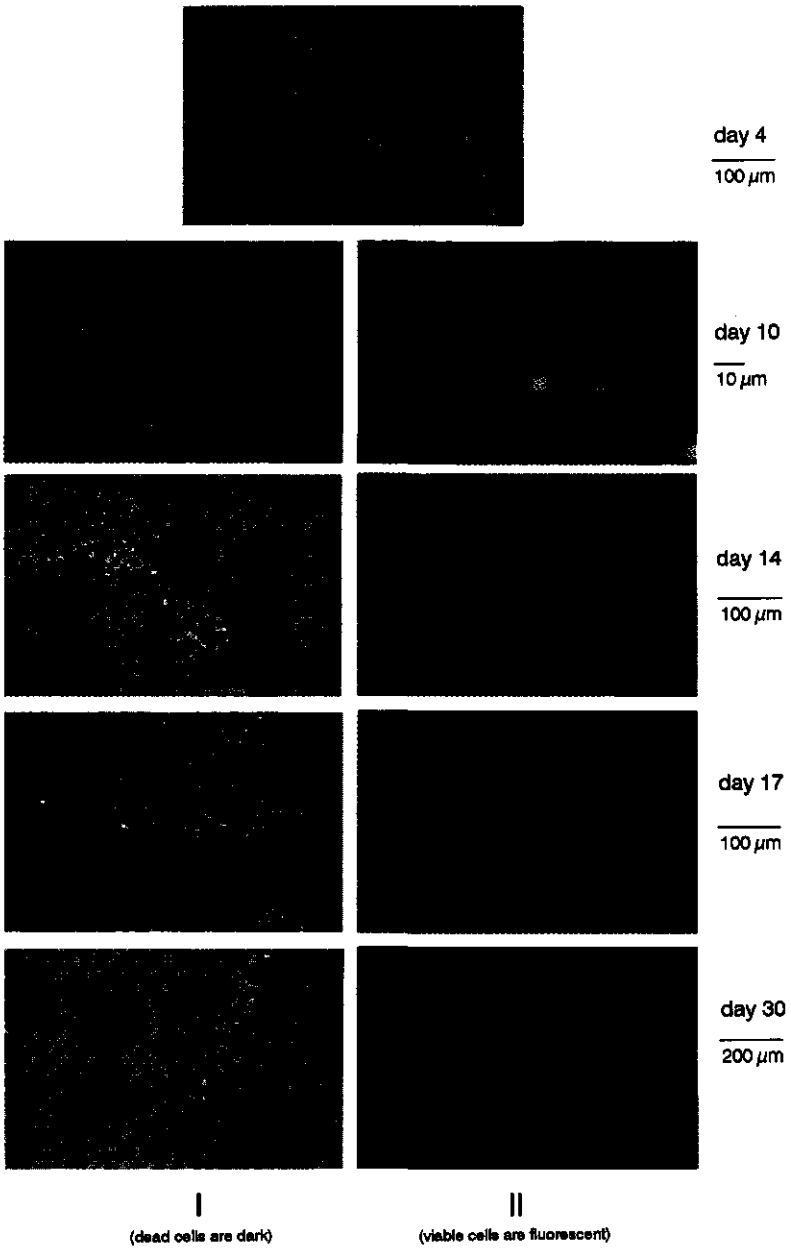

Figure 5A. Microscopic images of sections of carrageenan beads with immobilized *Nitrosomonas europaea* at day 4, 10, 14, 17 and 30.
I) image in bright field
II) image in UV light.

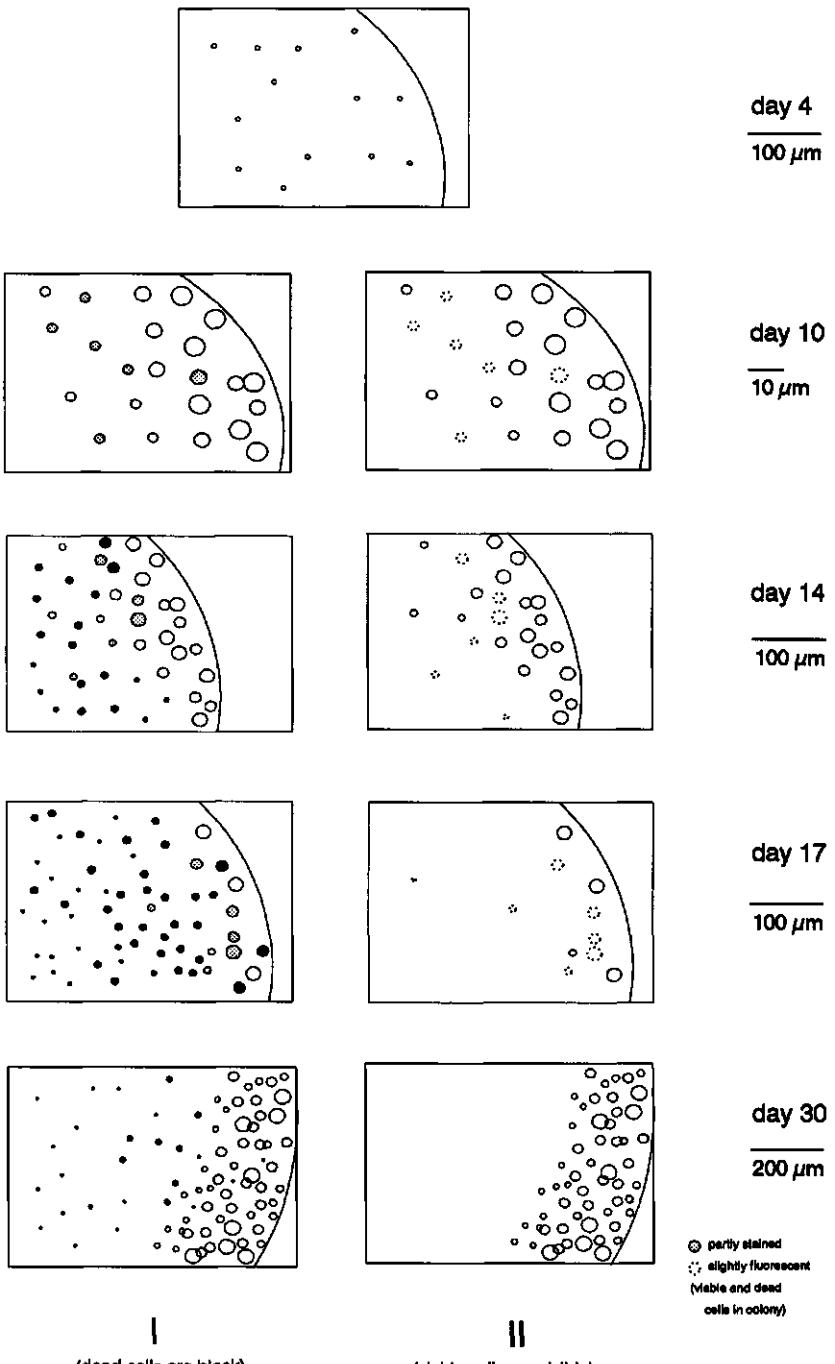

Figure 5B. Schematic representation of the images shown in Figure 5A.

In Figure 6 model simulations of the thickness of the biomass layer (just beneath the surface of the bead) are compared with the microscopically determined thickness. The model with biomass decay describes the experimental data better than the model without biomass decay, which predicts a thicker biomass layer than observed. These results show that death of immobilized *Nitrosomonas europaea* cells occurs and may play an important role. If fluctuations in substrate load occur regularly (e.g. daily fluctuations) than the effect of decay probably will be negligible. If these fluctuations, however, occur only a few times a year (e.g. seasonal or bimonthly fluctuations) the effluent quality, if biomass death has occurred, will decrease.

Biomass decay can be important for other immobilized-cell processes. In biofilm processes with multispecies, for instance, stratification of the different species in layers can occur as a result.

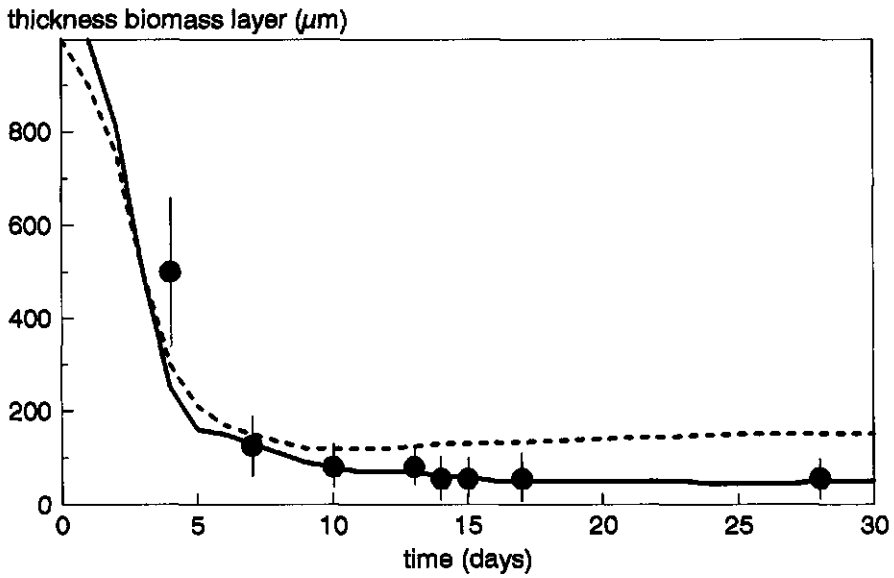

Figure 6. The microscopically determined thickness of the biomass layer just beneath the surface of the bead (●) in comparison with model predictions (——— with biomass decay; —— without biomass decay). A thickness of 1000 μm (half of the gel bead) is a homogeneous biomass distribution.

CONCLUSIONS

Biomass decay was found to be an important parameter in the dynamics of the studied immobilized-cell system. Different death rates were obtained for suspended *Nitrosomonas europaea* cells under oxygen or ammonium depletion. The death rate under ammonia depletion (4.97×10^{-6} s^{-1}; 31% of μ_{max}) was found to be approximately 5 times higher than under oxygen depletion (1.06×10^{-6} s^{-1}; 7% of μ_{max}) indicating that if problems occur during a run ammonium should be minimized to prevent decay of biomass.

Model simulations, reactor performance and microscopic analyses showed that immobilized *Nitrosomonas europaea* cells can loose their viability during cultivation. Cells in the centre of a bead die first, but at low substrate loadings also cells in the outer layers of a gel bead will die. If the substrate concentration is raised the number of viable cells near the surface of the gel bead increases again.

The FDA and lissamine-green staining techniques showed to be useful to distinguish between viable and dead cells in a carrageenan gel bead.

ACKNOWLEDGEMENT

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NOMENCLATURE

A	=	specific surface area of gel beads	m^{-1}
D	=	dilution rate	s^{-1}
$D_{gel,O2}$	=	diffusion coefficient of oxygen in the gel bead	$m^2.s^{-1}$
$D_{gel,NH4+}$	=	diffusion coefficient of ammonium in the gel bead	$m^2.s^{-1}$
k_d	=	decay rate of biomass	s^{-1}

$k_{d,O2}$	=	decay rate of biomass if no ammonium is present	s^{-1}
$k_{d,NH4+}$	=	decay rate of biomass if no oxygen is present	s^{-1}
$k_{l,O2}$	=	liquid\solid transfer coefficient for oxygen	$m.s^{-1}$
$k_{l,NH4+}$	=	liquid\solid transfer coefficient for ammonium	$m.s^{-1}$
K_S	=	substrate affinity constant for substrate	$mole.m^{-3}$
K_{O2}	=	substrate affinity constant for oxygen	$mole.m^{-3}$
K_{NH4+}	=	substrate affinity constant for ammonia	$mole.m^{-3}$
m	=	maintenance coefficient	$mole.kg^{-1}.s^{-1}$
r	=	position in the bead from the edge	m
r_s	=	macroscopic consumption rate of substrate	$mole.m^{-3}.s^{-1}$
r_x	=	biomass growth rate	$kg.m^{-3}.s^{-1}$
R	=	radius of the gel bead	m
S	=	substrate concentration	$mole.m^{-3}$
S_{bulk}	=	substrate concentration in bulk phase	$mole.m^{-3}$
S_{in}	=	substrate concentration in influent	$mole.m^{-3}$
S_{sur}	=	substrate concentration at surface of gel bead	$mole.m^{-3}$
t	=	time	s
X	=	biomass concentration	$kg.m^{-3}$
X_0	=	biomass concentration at t=0	$kg.m^{-3}$
X_t	=	biomass concentration at t=t	$kg.m^{-3}$
X_{max}	=	maximum biomass concentration	$kg.m^{-3}$
$Y_{x,s}$	=	yield coefficient of biomass on substrate	$kg.mole^{-1}$
μ	=	specific growth rate	s^{-1}
μ_{max}	=	maximum specific growth rate	s^{-1}

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CHAPTER 4

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.

Characteristics of and selection criteria for support materials for cell immobilization in wastewater treatment. E.J.T.M. Leenen, V.A.P. Martins dos Santos, K.C.F. Grolle, J. Tramper and R.H. Wijffels. Water Research 30 (1996): 2895-2996.

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 (De Gooijer *et al.*, 1991; Hunik et al, 1994[a]; Wijffels *et al.*, 1991; Wijffels and Tramper, 1995; Wijffels et al, 1995[a]). These studies have shown that high capacities can be reached, even under sub-optimal environmental conditions (Hunik *et al.*, 1994[b]; Leenen *et al.*, 1994; Wijffels *et al.*, 1995[b]). 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.*, 1993[a] and 1993[b]) 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.

Table I Characteristics and selection criteria of support materials for cell immobilization

Characteristic	Criterium
Solubility	**LOW**
Biodegradability	**LOW**
Stability	**HIGH** the support should have good mechanical and rheological properties,so that it is not sensitive to abrasion
Diffusivity	**HIGH**
Growth	**POSSIBLE** the micro-organisms must survive the immobilization procedure and grow in the support
Immobilization procedure	**SIMPLE** immobilization should be possible at large scale
Attachment of heterotrophic organisms	**MINIMAL**
Costs	**LOW**

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.

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 (Arnaud *et al.*, 1989; Audet *et al.*, 1990) 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 (Lewandowski *et al.*, 1987; Tramper and Grootjen, 1986; Van Ginkel *et al.*, 1983).

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.*, 1992[a]; 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; Asano *et al.*, 1992; Myoga *et al.*, 1991), 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.

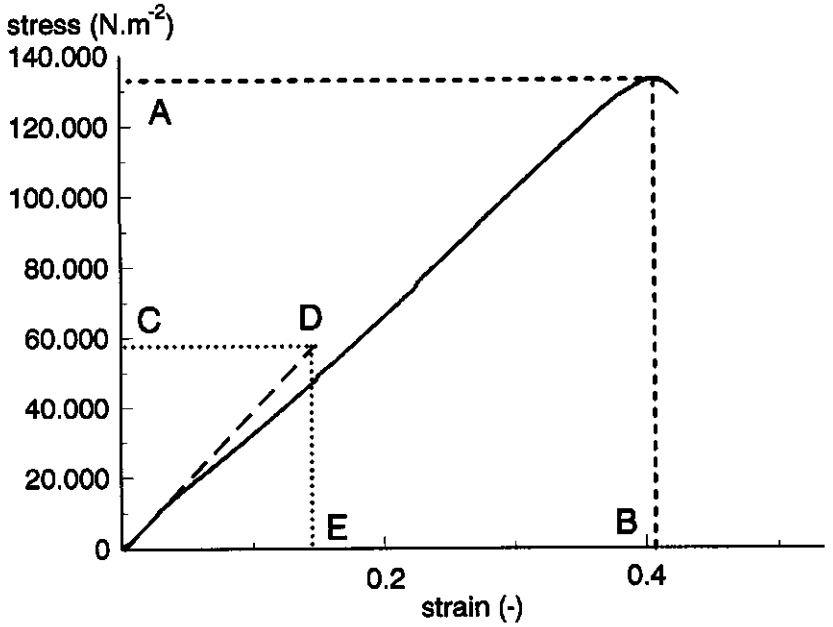


Figure 1. Stress-strain curve of 3% κ-carrageenan with a compression speed of $3x10^{-5}$ $m.s^{-1}$.

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 resistance to abrasion (gel strength) increases with the fracture stress (Sumino *et al.*, 1992[b]), the strain at fracture (deformation) and with decreasing rigidity modulus (Muscat *et al.*, 1993; Smidsrød and Christensen, 1991).

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 ($3x10^{-5}$ $m.s^{-1}$) 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, 1991[b]).

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.

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.

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.

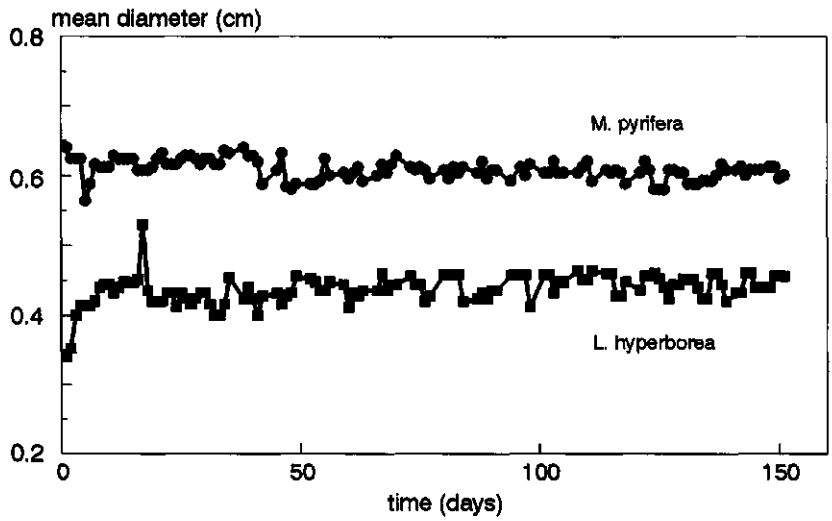


Figure 2. Solubility of Ba-Ca-alginate gel beads of *M. perifera* and *L. hyperborea* in demineralized water. Mean diameter of gel beads as a function of time.

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 many components are present. 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 life-time ranges from 3 to 40 days.

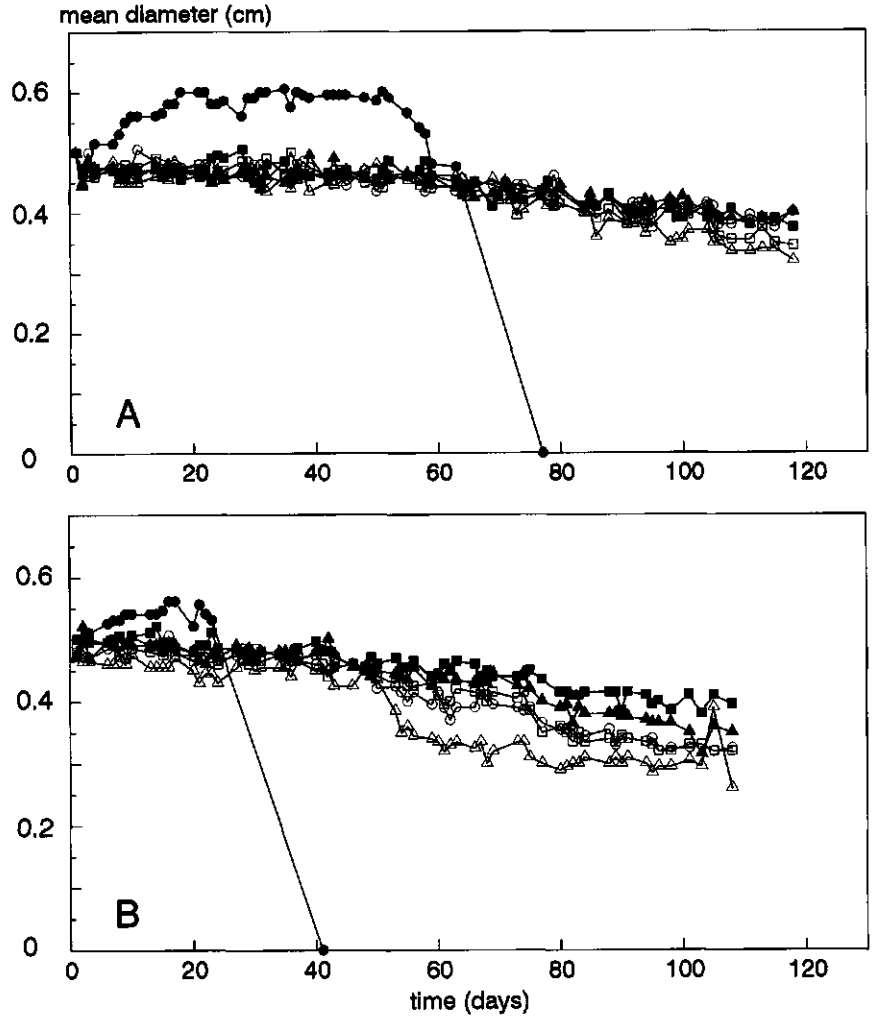


Figure 3. Solubility of Ba-Ca-alginate of *L. hyperborea* in
A) solution with different $NaCl/CaCl_2$ ratios.
B) solutions with different Na/Ca-phosphate ratios.
Mean diameter of gel beads as a function of time (● 10 mM Na^+; ■ 8 mM Na^+, 2 mM Ca^{2+}; ▲ 6 mM Na^+, 4 mM Ca^{2+}; ○ 4 mM Na^+, 6 mM Ca^{2+}; □ 2 mM Na^+, 8 mM Ca^{2+}; △ 10 mM Ca^{2+}).

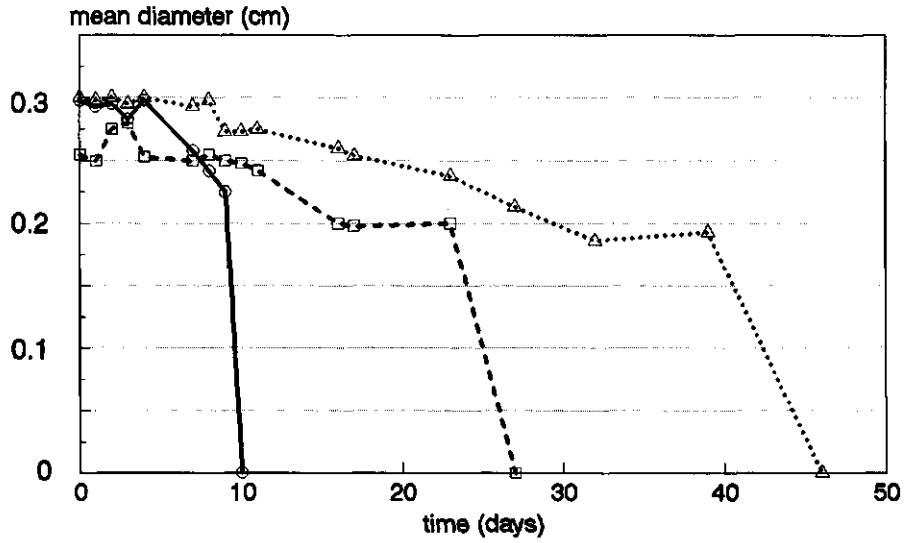


Figure 4. Solubility of Ba-Ca-alginate gels in domestic wastewater. Mean diameter of gel beads as a function of time (○ low; □ medium; △ high G content).

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 (Arnaud *et al.*, 1989; Audet *et al.*, 1990) 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.

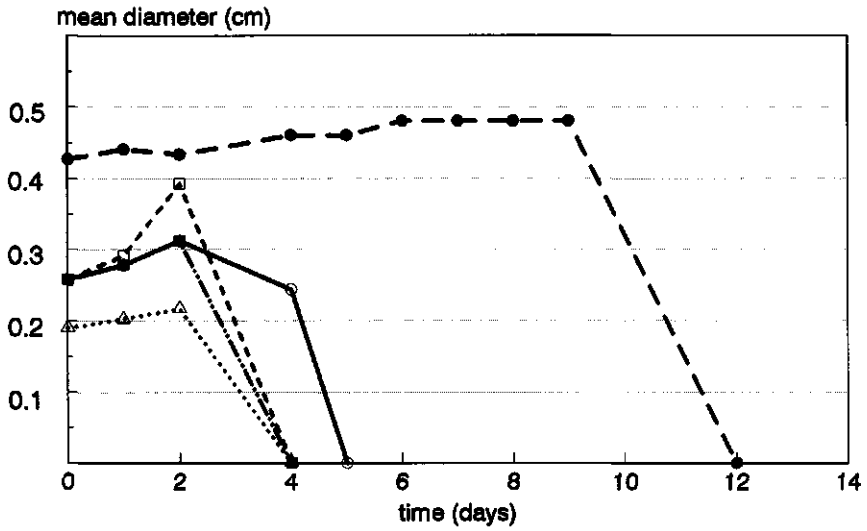


Figure 5. Solubility of κ-carrageenan-based gels in domestic wastewater. Mean diameter of gel beads as a function of time (○ pure; □ + $Al(NO_3)_3$; △ + arabic gum; ● + xanthan gum; ■ + locust bean gum).

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.

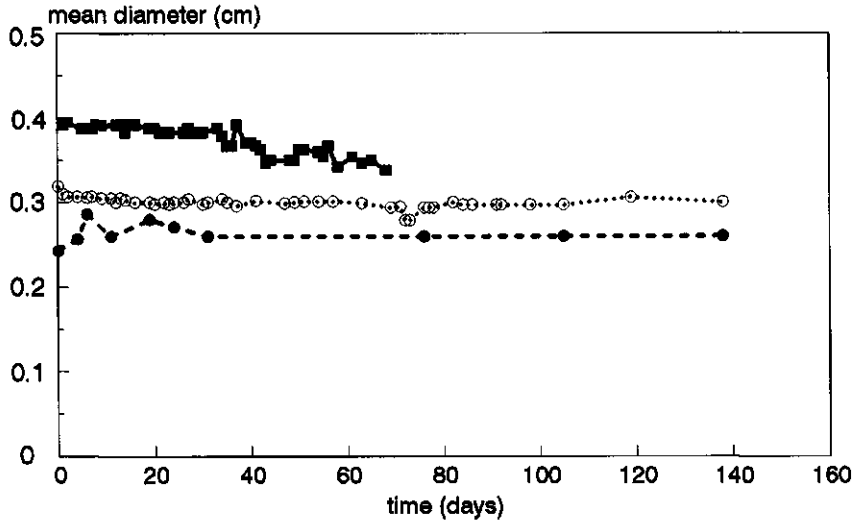


Figure 6. Solubility of synthetic gels in domestic wastewater. Mean diameter of gel beads as a function of time (○ PVA; ● PCS; ■ Polyacrylamide).

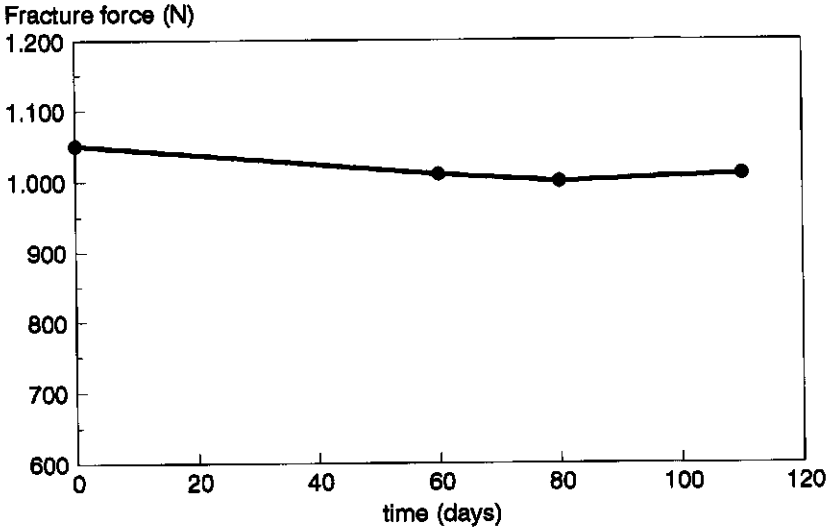


Figure 7. The fracture force of PVA gel beads (10% w/v, twice frozen and thawed) during exposure to domestic wastewater.

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.

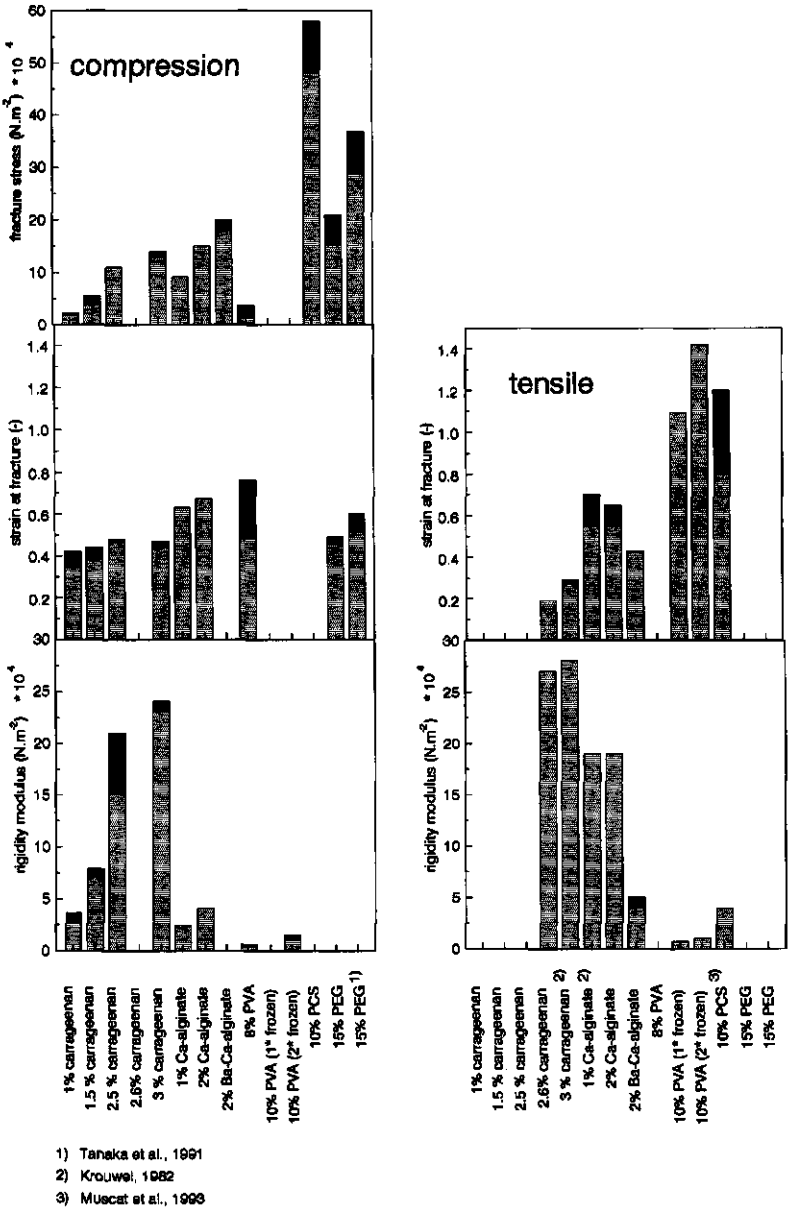
In marine environments several organisms (molluscs, bacteria, fungi) that contain carrageenan- or alginate-degrading enzymes were found (Østgaard 1993[a]; 1993[b]).

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. It is possible to biodegrade natural support materials, whereas 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.
Hunik (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.



1) Tanaka et al., 1991
2) Krouwel, 1982
3) Muscat et al., 1993

Figure 8. Fracture stresses, strains at fracture, and rigidity moduli of support materials. A) Compression tests; B) Tensile tests (⎯ standard error bar).

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 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} $x10^{-9}$ $m^2.s^{-1}$	$D_{glucose}$ $x10^{-9}$ $m^2.s^{-1}$	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 2x 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 (Hunik *et al.*, 1994[a]; Tramper and Grootjen, 1986; Van Ginkel *et al.*, 1983; Wijffels *et al.*, 1991).

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.

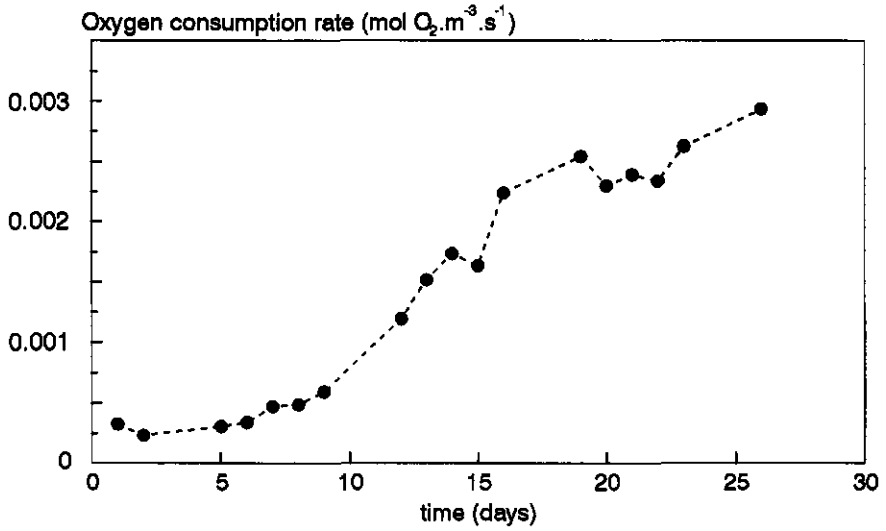


Figure 9. Growth of *Nitrosomonas europaea* in PCS. The oxygen consumption rate versus time.

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 (Takeshima *et al.*, 1993; Tanaka *et al.*, 1991).

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. 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.

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	laboriou s	laboriou s	simple

-	=	bad; not stable
+/-	=	moderate
+	=	good
++	=	very good
?	=	not determined or reported

Summing up, the synthetic gels (PVA, PCS and PEG), investigated in this study, are the more promising materials for application in wastewater-treatment systems.

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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 the most important property, beads having a similar fracture energy but 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. To determine this, 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. Agar beads were expected 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.

Relevance of rheological properties of gel beads for their mechanical stability in bioreactors. V.A.P. Martins dos Santos, E.J.T.M. Leenen, M.M. Ripoll, C. van der Sluis, T. van Vliet, J. Tramper, R.H. Wijffels, submitted.

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 (Tanaka and Nakajima, 1990), immobilized-cell processes are promising for wastewater-treatment purposes in general and for nitrogen removal in particular (Emori *et al.*, 1996; Nilsson *et al.*, 1980; Tanaka *et al.*, 1991; Wijffels *et al.*, 1993). Numerous studies have shown that high capacities can be reached in air-lift reactors containing (co-)immobilized (de)nitrifying cells (Kokufuta *et al.*, 1988; Nilsson *et al.*, 1980; Wijffels *et al.*, 1990). In some cases, such as that of Hitachi, Japan (Tada *et al.*, 1990), 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 (Leenen *et al.*, 1996). 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 (Leenen *et al.*, 1996). This is, however, mostly not the case both in aerated stirred tanks (Bucholz, 1979; Nienow and Conti, 1978;

Poncelet and Neufeld, 1989) and in air-driven reactors (Gjaltema *et al.*, 1995; Hunik and Tramper, 1991). 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 vicinity of the stirrer (Cherry and Papoutsakis, 1986; Kusters, 1991) whereas in air-driven reactors the shear fields are comparatively more homogeneous (Merchuk, 1990; Merchuk and Berzin, 1995). This results in local shear stresses that are generally lower than those present in stirred tanks at the same power input (Chisti, 1989). Nevertheless, severe abrasion of biocatalyst particles has been reported to occur in such reactor systems (Gjaltema *et al.*, 1995; Hunik and Tramper, 1991). Mechanical stresses can manifest 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, 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 reported (Arnaud *et al.*, 1989; Guiseley, 1989; Martinsen *et al.*, 1989; Skjåk-Bræk *et al.*, 1989). 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 (Cheetham *et al.*, 1979; Klein and Eng, 1979).

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 discussed shortly below. Furthermore, the possible shear stresses that could have been present in the reactors were calculated. For explanation of the symbols see the Nomenclature section. All values were estimated for the experimental conditions described in the Materials and Methods section. The average energy dissipation was calculated according to the expression (Chisti, 1989):

$$\langle \varepsilon \rangle = \frac{F_g}{mV} RT \ln\left(\frac{P_s}{P_t}\right) \quad (1)$$

The average collision rates for each of the ranges was calculated assuming isotropy, i.e., that the local energy dissipation rate (ε) is equal to the average one ($<\varepsilon>$). In the experiments described $<\varepsilon> = 0.54$ 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 (1964) 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 (1990), Kawase and Moo-Young (1990), Cherry and Papoutsakis (1986) and Baldyga and Bourne (1995). Baldyga and Bourne (1995) estimate the average and maximum pressure (stress) difference acting on a bead with the following expressions:

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

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

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 and Tramper (1991) 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 may 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 and Vlak (1988):

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

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

Collisions between particles As described above, in any agitated reactor, at every moment, eddies in a broad range of sizes are always present. 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 (1991) 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 (1991), Spielman (1978) and Hinze (1971). 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 (Van den Bijgaardt, 1988). Combining the estimation of the collision frequency according to Kusters (1991) with the expression for an elastic head-on collision (Van den Bijgaardt; 1988) the maximum compression stress for all collisions can be calculated by $p_{max} = \Delta\rho^{2/3}\ \Delta v^{4/3}\ E^{1/3}$ (Van den Bijgaardt, 1988). 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 (Van Vliet and Peleg, 1991)

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.

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 (τ_f, in Figure A or G) and strain (ε_f, in Figure 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 (e.g. 0CB or 0GH in Figure) 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. Only for (very) small deformations it is independent of it and representative for the undisturbed material.

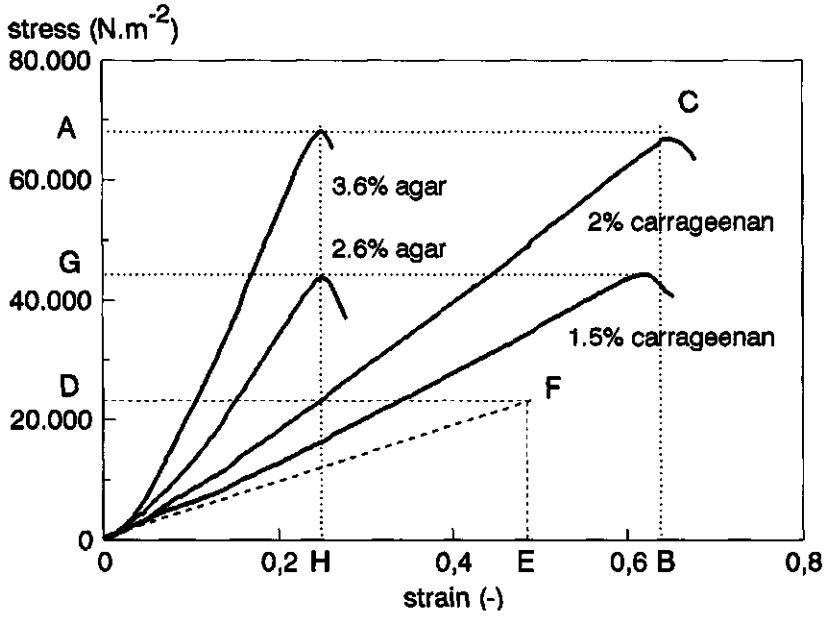

Figure 1: Stress-strain curves of 4 gels (1.5% carrageenan, 2% carrageenan, 2.6% agar and 3.6% agar) at a compression rate of 3 mm.min^{-1}.

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 (Klein and Eng, 1979; Krouwel, 1982; Leenen *et al.*, 1996; Nussinovitch *et al.*, 1994; Sumino *et al.*, 1992), and with decreasing compression or tension modulus (Muscat *et al.*, 1993; Smidsrød and Christensen, 1991). Thereby, it is implicitly assumed that if the biocatalyst particle is mechanically strong, it will be also resistant to abrasion. This has not been proven 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 (Skjåk-Bræk *et al.*, 1989), 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 (Fig. 1), beads of these four gels were made 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 (Emori *et al*, 1996) 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 specimen, 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 and Peleg (1991) and Luyten (1988) 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 to 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.* (1989). 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 (Skjåk-Bræk *et al.* 1989). 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 the boiling point was reached. 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: $A_t = A_0 * h_0 / ht$, 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 Δh 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 (τ_f) on the Y-axis and the accompanying fracture strain (ε_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 (Van Vliet *et al.*, 1992):

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

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, Student's t-tests with 99% and 95% confidence intervals were executed.

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 (1946). 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 of medium and 560 ml of 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 *et al.* (1956) and modified as suggested by Halliwell *et al.* (1983). This spectrophotometric method is based on the development of an orange-yellow colour when polysaccharides containing (potentially) free reducing groups are first treated 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. For 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.

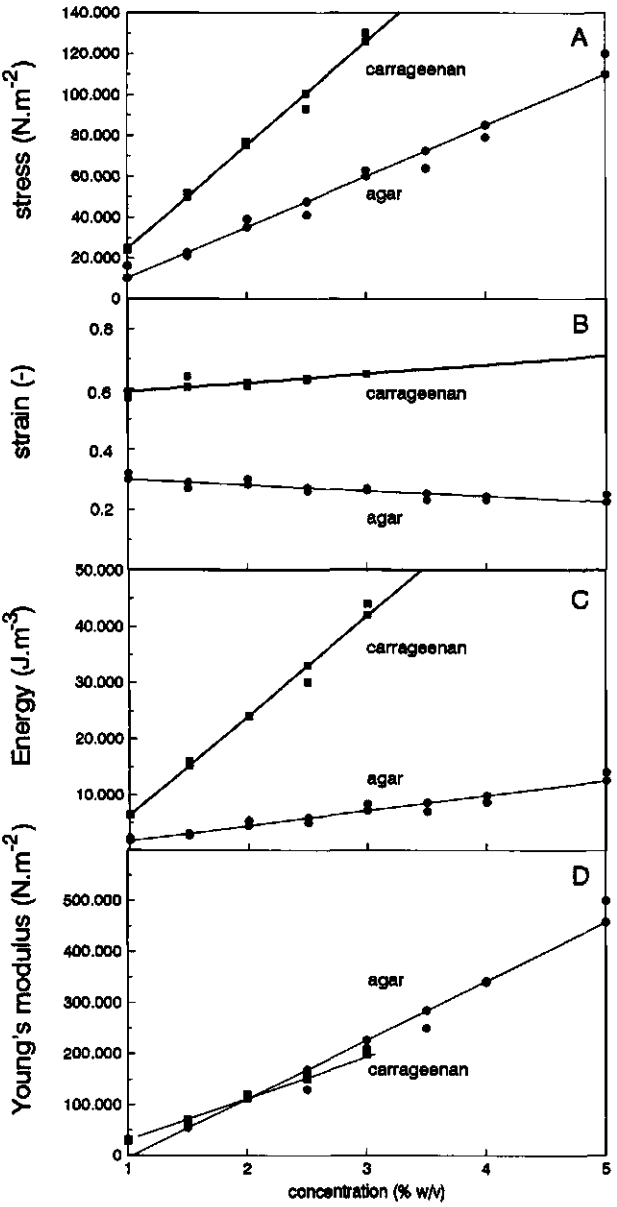

Figure 2. Rheological properties of carrageenan and agar gels as a function of polymer concentration (% w/v). a) stress at fracture; b) strain at fracture; c) fracture energy; d) Young's modulus. Compression speed of 3 $mm.min^{-1}$.

PEG (15% w/v) fractures at a stress of 139770 $N.m^{-2}$ and a strain of 0.437;

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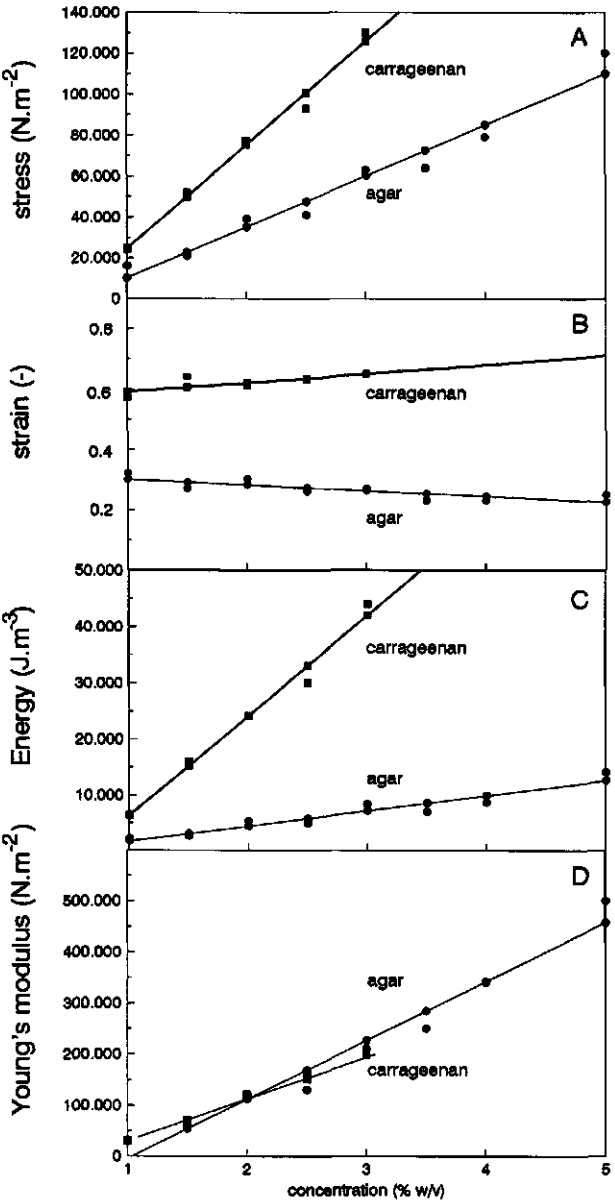

Figure 2. Rheological properties of carrageenan and agar gels as a function of polymer concentration (% w/v). a) stress at fracture; b) strain at fracture; c) fracture energy; d) Young's modulus. Compression speed of 3 $mm.min^{-1}$. PEG (15% w/v) fractures at a stress of 139770 $N.m^{-2}$ and a strain of 0.437; the fracture energy is 27154 $J.m^{-3}$; the Young's modulus is 264750 $N.m^{-2}$.

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. 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 *et al.* (1992). Therefore, conclusions about the way a material will fracture can not be based on the measurement of such small-deformation properties, nor can predictions on its resistance to abrasion in a reactor be made (see Abrasion experiments below).

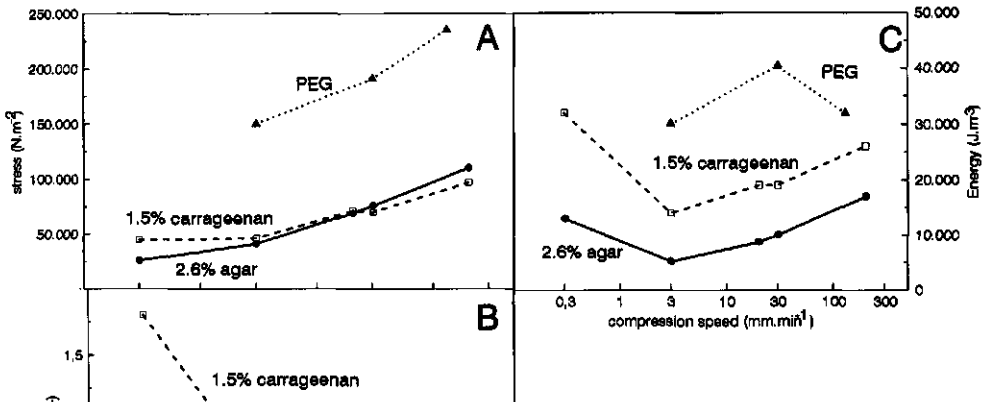

Figure 3. Rheological properties of 1.5% carrageenan, 2.6% agar and 15% PEG gels as a function of deformation rate (compression speed). a) stress at fracture; b) strain at fracture; c) fracture energy.

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.

Table I. Fracture properties of the selected carrageenan and agar beads.

	n	τ_f (N.m^{-2})	ε_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 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.

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.

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 $\%_{gel\ abraded}$ / $mm^2_{gel\ bead}$ 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.

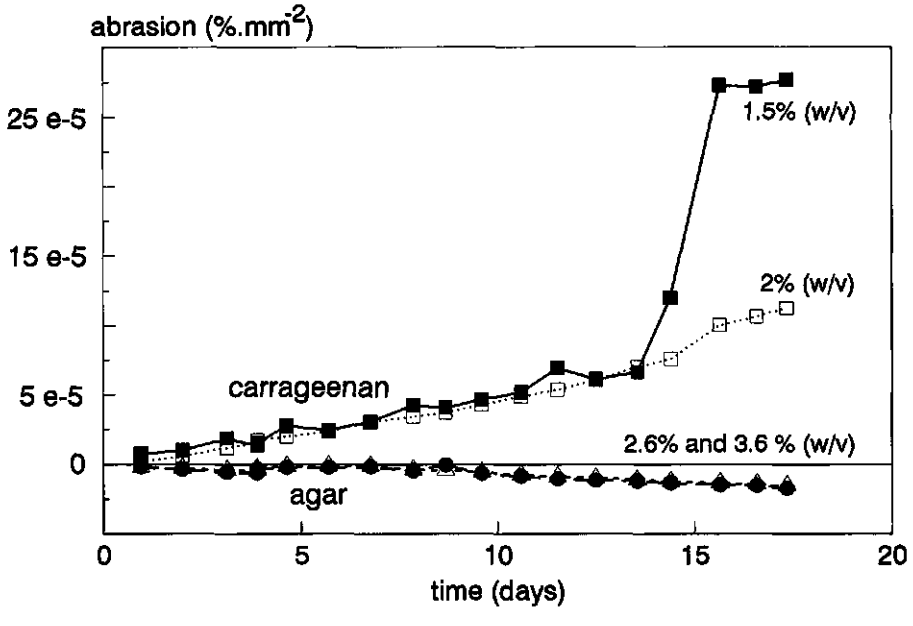

Figure 4. Abrasion of carrageenan and agar beads in bubble columns. The $\%_{gel\ abraded}.mm^{-2}_{gel\ bead}$ in time.

Agar gels did not abrade during the 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.

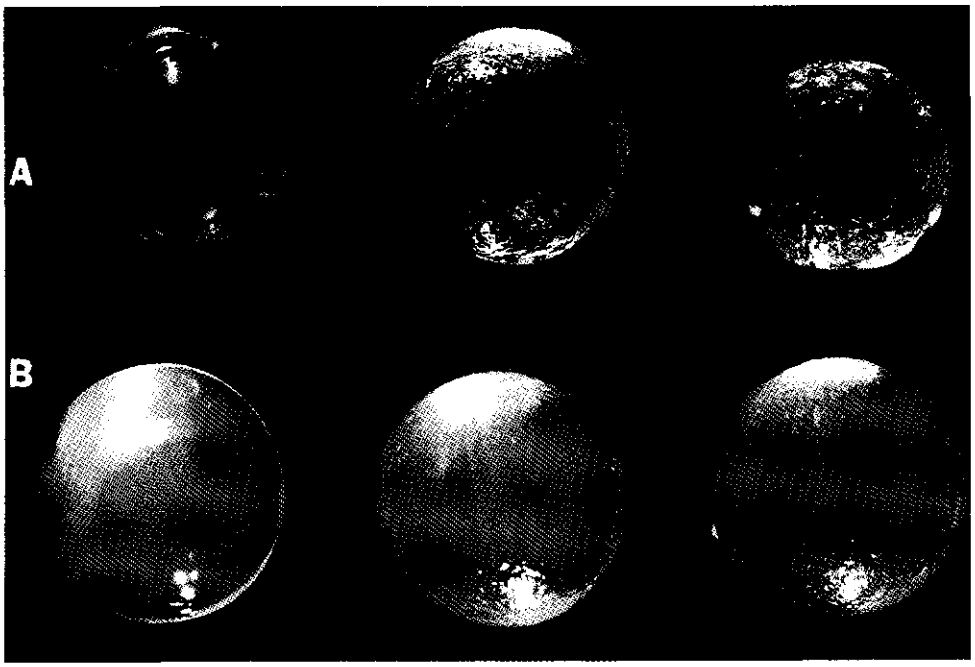

Figure 5. The appearance of 1.5% carrageenan (A) and 2.6% agar beads (B) at day 0, day 10 and at the end of the abrasion experiment.

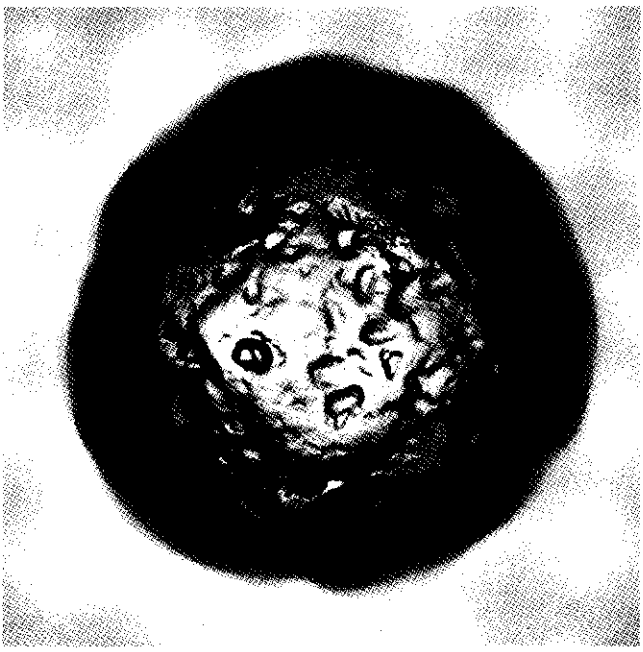

Figure 6. A typical image of a 1.5% carrageenan bead at the end of the abrasion experiment.

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 had lower stress at fracture than those of carrageenan.

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: 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 repeated application of stresses to the bead surface.

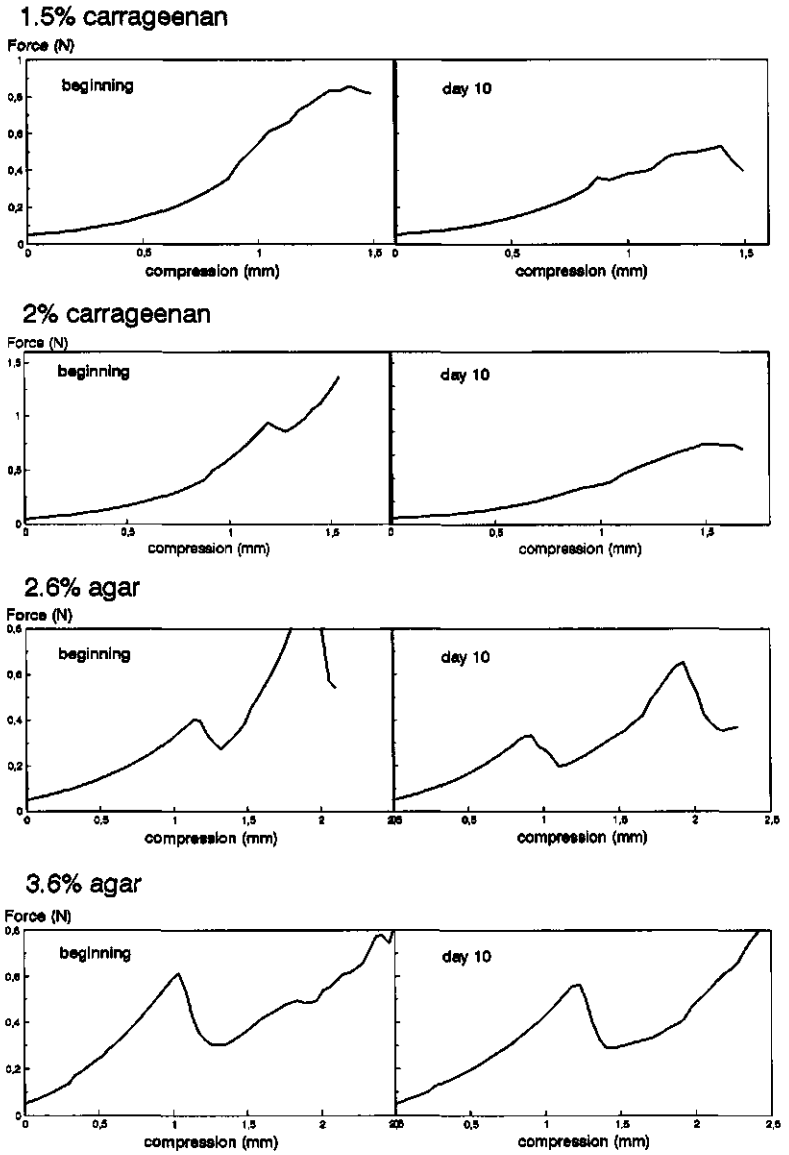

Figure 7. Stress-strain curves for carrageenan and agar beads at day 0 and day 10 of the abrasion experiment.

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 (Figure 9). A constant weight (100 g) 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.

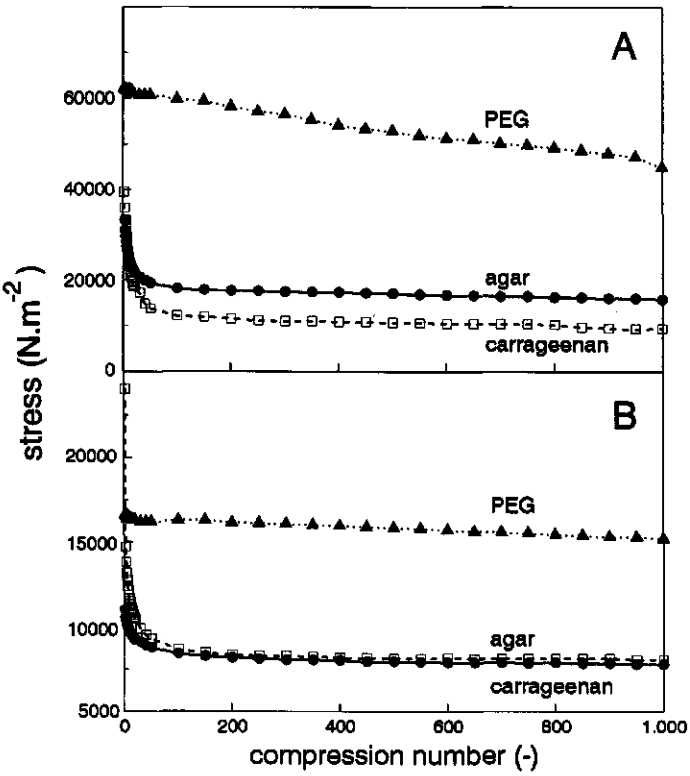

Figure 8. The evolution of the resistance of compression (stress) of 1.5% carrageenan, 2.6% agar and 15% PEG samples at a) 75% and b) 22.8% of their maximum compression. Compression rate of 3 mm.min^{-1}.

Carrageenan gels deformed more than those of agar and these much more than PEG samples. 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 (Takeshima *et al*, 1993). 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.

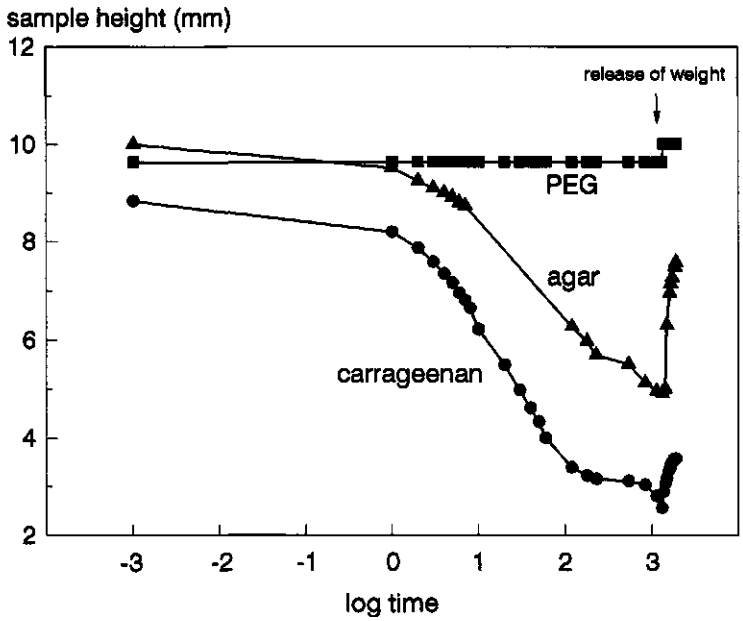

Figure 9. Compression of carrageenan, agar and PEG gels with a constant force of 3124 $N.m^{-2}$. After 33 hours the force was removed and the recovery determined.

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 (Gordon, 1968; Luyten, 1988; Van Vliet *et al.*, 1991). 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 (Atkins and May, 1985; Luyten, 1988; Van Vliet *et al.*, 1991). What is generally noticed as macroscopic fracture is the moment/region that the crack propagates spontaneously. Slow local growth of microcracks (irregularities), caused by the local stresses at a crack tip being 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 (Atkins and May, 1985; Luyten, 1988; Van Vliet *et al.*, 1991). 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. 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 (Van Vliet *et al.*, 1992).

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 (Hüsken *et al.*, 1996; Wijffels *et al.*, 1995). 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 (Harremoes *et al.*, 1980; Krouwel, 1982). A colony of cells will form a weak spot and can strongly decrease its fracture stress (Van Vliet and Walstra, 1995). Indeed, Nussinovitch *et al.*, (1994) have shown that both the deformability and strength of agar and alginate beads decreased considerably for high biomass concentrations.

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NOMENCLATURE

A_0	initial area	m^2
A_t	actual bearing area	m^2
c	relative vertical change in radius due to compression	m
C_p	constant according to Baldyga and Bourne (1995)	-
d_b	diameter gel beads	m
d_f	thickness of the liquid film	m
E	Youngs modulus	$N.m^{-2}$
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 and Bourne, 1995)	
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	$N.m^{-2}$
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 downstream side of a resistance	$m^2.s^{-1}$
Δv	velocity difference between two colliding particles	$m^2.s^{-1}$
V	molar volume of the gas for a given temperature	$m^3 mol^{-1}$
$<\varepsilon>$	mean energy dissipation rate per unit mass of liquid	$m^2.s^{-3}$
ε_f	strain at fracture	-
ε_H	Hencky strain	-
γ	strain rate	s^{-1}
$<\lambda>$	size of smallest eddies (Kolmogoroff scale) when energy dissipation rate is $<\epsilon>$	m
ρ	medium density	$kg.m^{-3}$
$\Delta\rho$	density difference between particle and medium	$kg.m^{-3}$
τ	shear stress	$N.m^{-2}$
$<\tau>$	shear stress when energy dissipation rate is $<\epsilon>$	$N.m^{-2}$
τ_f	stress at fracture	$N.m^{-2}$

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CHAPTER 6

ARTIFICIALLY IMMOBILIZED MICROORGANISMS FOR NITRIFICATION OF WASTEWATER

ABSTRACT

The feasibility of artificially immobilized nitrifying bacteria for the treatment of domestic wastewater was investigated. Firstly, suitable synthetic supports were selected. For this, the possibility of cells to grow in the support as well as the mechanical stability of the supports was investigated. Secondly, a suitable support was used to entrap *Nitrosomonas europaea* and *Nitrobacter agilis* cells and a nitrification experiment in pretreated domestic wastewater was done.

Successful nitrification was accomplished with this process. Heterotrophic growth did not occur, but a film of attached ciliates was observed on the bead surface. These ciliates did not decrease the nitrification activity.

Nitrification by artificially immobilized microorganisms in wastewater.
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INTRODUCTION

Lately, regulations regarding effluent concentrations, e.g. of nitrogen compounds, for wastewater-treatment plants are becoming more stringent. Nitrogen compounds cause eutrophication of the surface water, which may result in massive algal growth and subsequently fish mortality. Nitrification, defined as the biological oxidation of ammonia to nitrate via nitrite has been studied for a long time (Painter 1986; Prosser, 1986 and 1989; Sharma and Ahlert, 1977). Various autotrophic microbial species carry out the two oxidation reactions. *Nitrosomonas* spp. carry out the ammonia oxidation and *Nitrobacter* spp. are responsible for the nitrite oxidation. The growth rate of these bacteria is low compared to heterotrophic bacteria, with which they have to compete for oxygen. In activated-sludge plants, both availability of oxygen and the slow growth rate are the reasons that it is difficult to obtain and maintain sufficient nitrifying bacteria.

An efficient method to retain slow-growing bacteria in continuous-flow reactors is by immobilization. Immobilized-cell reactors can be compact in comparison to activated-sludge plants, because the residence time of the liquid phase can be chosen independently from the specific growth rate of the nitrifying organisms. Several fundamental aspects of artificially immobilized nitrifying bacteria were studied earlier (Hunik *et al.*, 1994; Leenen *et al.*, 1994; Wijffels *et al.*, 1991) and high nitrification rates could be reached in mineral media (Wijffels *et al.*, 1993).

In previous research κ-carrageenan or calcium-alginate were used as immobilization materials. These materials are not suitable for application in wastewater treatment, because they are fragile (Hunik, 1993) and dissolve if the proper counter ions for the gel matrix are not available (Smidsrød and Skjåk-Bræk, 1990). Some synthetic supports (e.g. polyvinyl alcohol, polycarbamoyl sulphonate, polyethylene glycol) have shown to have interesting characteristics for application in wastewater-treatment systems (Leenen *et al.*, 1996).

In the research described in this study, these promising synthetic supports have been investigated further. For this, the survival and growth of nitrifying cells in the support was determined and the sensitivity to abrasion was studied with oscillation experiments. Subsequently, one of these promising supports was chosen to immobilize nitrifying cells and a nitrification experiment in wastewater was executed. To minimize the effect of heterotrophic organisms, pretreated wastewater with a low chemical oxygen demand (COD) was used.

MATERIALS AND METHODS

Chemicals and wastewater

Polyvinyl alcohol (PVA; 70000-100000 A. Mol. Wt.) was obtained from Sigma, USA, Chemically crosslinked polyethylene oxide (PEO) was synthesized by Holland Biomaterials Group (HBG) in Enschede, The Netherlands. Polycarbamoyl sulphonate (PCS) was obtained from the Federal Agricultural Research Centre (FAL), Institute of Technology, Braunschweig, Germany; sodium alginate Manucol DM was from Kelco. All other chemicals used were from Merck, Darmstadt, Germany.

Domestic wastewater from Bennekom (Department of Environmental Technology, Wageningen Agricultural University, technological plant) was used. The wastewater was pretreated in a high-loaded activated-sludge plug-flow reactor in which dephosphorisation took place and most of the organic substrate (COD) was removed. The pretreated wastewater contained per m^3 approximately 70 g COD and 3 mole (54 g) ammonium.

Microorganisms

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 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 $NaHCO_3$.

Nitrobacter agilis (ATCC 14123) was cultivated in batch (2 dm^3) at 30°C. The medium contained per m^3 of demineralized water: 20.0 mole KNO_2; 0.20 mole $MgSO_4$; 2.44 mole NaH_2PO_4; 7.56 mole Na_2HPO_4; 1 mmole Na_2MoO_4; 0.015 mmole $ZnSO_4$; 0.016 mmole $CuSO_4$; 0.018 mmole $FeSO_4$ and 5 mmole $CaCl_2$. The pH of the medium was adjusted to 7.8 with 2 N KOH.

Immobilization procedures

Just before immobilization, a concentrated cell suspension of *Nitrosomonas europaea* or a mixed suspension of *Nitrosomonas europaea* and *Nitrobacter agilis* cells were mixed with the polymer solution.

Polycarbamoyl sulphonate. Immobilization in PCS was done according to Vorlop *et al.* (1992) and Muscat *et al.* (1993). A 40% (w/w) PCS solution was mixed with a 0.5 M $CaCl_2$ solution to a PCS content of 10% (w/w). The pH was adjusted to 6.2 after which the cell suspension was added. This suspension was dripped into a 0.75% (w/v) alginate solution (pH 8.5). Immediately a thin calcium-alginate layer, surrounding the PCS drop, was formed by ionotropic gelation. Gelation of PCS is much slower and takes place in the alginate capsule at pH 8.5. After the PCS-gelation (approximately 30 min.) the alginate layer was dissolved in a 0.1 M sodium phosphate buffer. The obtained PCS gel beads had an average diameter of 2 mm.

Polyvinyl alcohol. PVA gel was produced by iterative freezing and thawing (Ariga *et al.*, 1987). PVA was mixed with demineralized water and heated to 70°C. After the PVA was dissolved the solution was cooled to 30°C and mixed with a concentrated cell suspension of *N. europaea*. Cryoprotectant (concentrations and types see below) dissolved in demineralized water was added and a final PVA concentration of 10% (w/w) was reached.

To obtain different freezing rates this mixture was extruded into different solutions.The solutions used were: liquid N_2 (high freezing rate; 200°C.min^{-1}) and paraffin of -20°C (medium freezing rate; 20°C.min^{-1}). A low freezing rate (0.2°C.min^{-1}) was obtained by adding 1% alginate (Manucol) to the mixture and extruding this into a 0.75 M $CaCl_2$-solution. After this the derived beads were frozen overnight in a freezer (-33°C) and the next days thawed at 5 or 30°C. The obtained PVA beads had in all cases an average diameter of 2 mm.

Polyethylene oxide. Immobilization in PEO (Holland Biomaterials Group, Enschede, The Netherlands) was done by mixing the prepolymer solution with an equal volume of cell suspension. Hereafter the polymerization was started by adding an initiator and by mixing thoroughly. The suspension was poured into small grooves in a glass plate. After approximately 5 hours the polymerization was complete and the 'spaghetti-like' gels were cut into equal pieces. After adding these pieces to growth medium or demineralized water the pieces swelled 3 to 4

times, resulting in small cylindrical particles with a diameter and height of both 2 mm.

Growth

Nitrosomonas europaea cells immobilized in PEO (50 ml gel beads) were incubated in an air-lift loop reactor with 2.5 dm^3 growth medium with 20 mM $(NH_4)_2SO_4$. *Nitrosomonas cells* immobilized in PVA (2 ml gel beads) were incubated shaking in 50 ml growth medium at 30°C. Periodically the activity (see below) was measured.

Growth of nitrifying cells in PCS was investigated and reported earlier (Leenen *et al.*, 1996).

Activity assay

Oxygen consumption rates measured in a Biological Oxygen Monitor (Yellow Springs Instruments, Ohio, USA) can be used as an estimation of the overall viable biomass (Van Ginkel *et al.*, 1983). In an 8 cm^3 vessel a fixed number of gel beads with immobilized cells were added together with 4 cm^3 50 mM phosphate buffer (pH 7.5) and the suspension was aerated with oxygen for 10 min. Then the vessel was sealed with the oxygen electrode (model 5331, Yellow Springs Instruments, Ohio, USA) such that no air bubbles remained in the liquid. Substrate was added through the seal with an analytical syringe (0.1 cm^3); a high concentration was used to avoid a significant change in the liquid volume. The decrease in oxygen concentration (between 100-80% oxygen saturation) was then recorded as a function of time. These assays were done at 30°C. The substrate used was 1 M $(NH_4)_2SO_4$ for *Nitrosomonas europaea*.

In the pretreated wastewater a small amount of COD was present. To check if heterotrophic biomass was accumulating on the gel beads activity assays with 1 M CH_3COONa as substrate were done. The activity was defined as the initial oxygen consumption rate of the gel beads and was expressed as mole $O_{2,consumed} \cdot s^{-1} \cdot m^{-3}_{gel}$.

Effect of cryoprotectants on activity of suspended *Nitrosomonas europaea*

Activity assays were done after one time freezing and thawing of the suspended cells in the presence of several cryoprotectants. The cryoprotectants and concentrations (w/w) used were: 2.5 and 10% glycerol; 0.5 and 2.5% methanol; 0.5 and 2.5% ethanol; 0.5, 2.5 and 10% ethylene glycol; 5.0% adonitol; 0.3 M sorbitol; 0.3 M trehalose; 0.3 M sucrose; 0.3 M fructose; 0.3 M lactose; 0.3 M glucose; 0.1, 0.15 and 0.3 M maltose. These cryoprotectants were added with a 50 mM phosphate buffer (pH 7.5). In some cases the saturation concentration of oxygen in these mixtures was different than the reference experiment. This difference was measured and corrected for. Hundred percent activity was set to be the activity of the cell suspension of *Nitrosomonas europaea* before freezing and thawing without the addition of a cryoprotectant.

Stability

The stability of supports was measured by determining the resistance to compression during several oscillations (simulated collisions), as was described by Martins dos Santos (*et al.*, 1996). Compression was done with a 2000 N load cell with a constant speed ($3x10^{-5}$ $m.s^{-1}$) at room temperature (20°C). First the force and relative deformation (strain) at the fracture point were determined. After this, oscillation experiments were done at 23% and 75% of the maximum deformation.

Test piece preparation. The same procedure as for the immobilization of cells was used, except that demineralized water or phosphate buffer (pH 7.5) was used instead of the cell suspension and that cylinders with a diameter of 15 mm and a height of 10 mm were used.

Nitrification in wastewater

The *Nitrosomonas europaea* and *Nitrobacter agilis* cells coimmobilized in PCS were cultivated in a continuous-flow internal air-lift loop reactor (0.45 dm^3). The experimental set-up is shown schematically in Figure 1.

Initially the reactor was fed with the same mineral medium as used for the cultivation of the pure culture of *Nitrosomonas europaea*, except that Na_2MoO_4 (1 mmole. m^{-3}) and $ZnSO_4$ (0.015 mmole. m^{-3}) were added as trace elements for the growth of *Nitrobacter agilis*. After growth was observed the mineral medium was gradually replaced by the pretreated wastewater. This was done to have a slow decrease in ammonium concentration. After 4 days the reactor was fed with 100% pretreated domestic wastewater with a hydraulic retention time of 18.4 min. At day 25 the retention time was increased to 23.2 min. The cultivation was done at 30°C with a 7% (v/v) gel load and a superficial gas-flow rate of 1.2×10^{-3} $m.s^{-1}$. The pH was controlled to 7.5 with 7 M $KHCO_3$.

After feeding the reactor with pretreated wastewater for 45 days the influent was changed to mineral medium again.

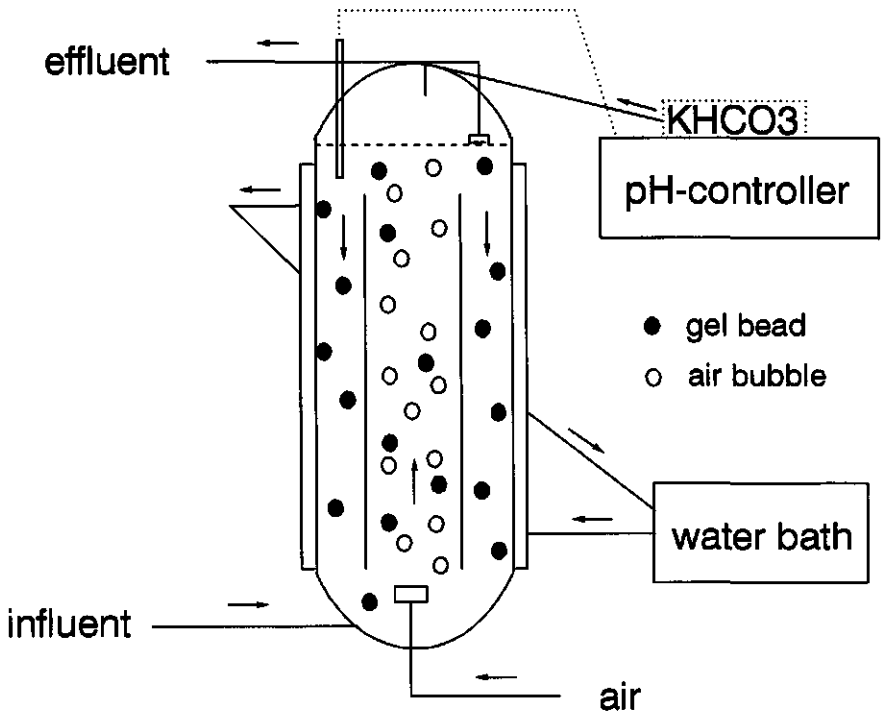

Figure 1. Internal air-lift loop reactor.

Analyses

The NH_4^+, NO_2^-, and NO_3^- concentrations in influent and effluent were determined spectrophotometrically using an auto-analysis system (Skalar 5100; Greenberg *et al.*, 1985).

The COD concentrations in influent and effluent were measured daily with the micro-COD technique (Jirka and Cater, 1975). Ag^+ was used as a catalyst for the oxidation of the organic matter at low pH with potassium dichromat. The reduced chromate concentration is a direct measure for the COD concentration in the sample.

Microscopy

The whole gel beads were examined under an inverted microscope (Olympus CK2) after 3 weeks and at the end of the experiment (at day 44). At day 44 photographs were taken of the whole beads and of parts of the film formed on these beads (magnification 40x, 100x, 200x). The photographs were taken with a Kodak Ektachrome film (100 ASA).

RESULTS AND DISCUSSION

Growth

Cells have to survive the immobilization procedure to be able to grow in the formed support. The procedure to prepare synthetic gels are generally hostile and often cause cell death. Therefore, the growth of *Nitrosomonas europaea* in PCS, PVA and PEO was investigated.

Previously, we reported that growth in PVA was problematic (Leenen *et al.*, 1996). An attempt to optimize the iterative freezing and thawing procedure was done by adding a cryoprotectant to the cell polymer suspension before freezing. First the effect of several cryoprotectants on the residual activity of suspended *Nitrosomonas europaea* after a single freeze and thaw step was determined at

high and low freezing and thawing rates (see Table I). The residual activity decreased drastically in almost all cases. Different cryoprotectants resulted in different protection of the cells. Disaccharides, for instance, protected the cells the best at a high thawing rate almost independently from the freezing rate, while the use of polyalcohols resulted in higher activities at a low freezing rate. The highest residual activities were obtained with disaccharides (trehalose, sucrose, lactose and maltose) as cryoprotectant.

Adding a cryoprotectant to the cell polymer suspension can alter the characteristics of the resulting gel formed. Therefore, the effect of cryoprotectants on the gel strength was determined qualitatively by squeezing the gel after it was formed. The results of these observations are also reported in Table I.

Table I: Effect of cryoprotectants on the residual activity of suspended *N. europaea* cells after one time freezing and thawing and the effect of cryoprotectants on the strength of the PVA gel.

	residual activity (%)*				
Cryoprotectant	HF-HT	HF-LT	LF-HT	LF-LT	Gel strength#
none	20-22	9-11	35-38	22-24	reference
methanol	0	0	0	0	=
ethanol	5-6	8-10	4-5	10-12	=
glycerol	14-16	8-10	27-19	46-52	=
adonitol	33-35	15-16	34-36	38-40	+
sorbitol	7-8	6-7	36-38	33-34	=
glucose	12-13	8-9	22-23	18-20	-
fructose	10-11	6-7	29-31	27-29	-
lactose	59-61	32-34	62-64	43-45	--
maltose	60-63	33-35	64-67	47-50	--
sucrose	56-59	36-37	68-69	40-44	-
trehalose	76-80	40-44	70-75	53-55	--
ethylene glycol	11-13	6-7	8-9	22-24	=

* HF = high freezing rate (200°C.min^{-1}) LF = low freezing rate (0.2°C.min^{-1})
HT = high thawing rate (10°C.min^{-1}) LT = low thawing rate (0.25°C.min^{-1})

\# = same gel strength as reference
+ stronger than reference
- weaker than reference
-- no gel formed

The addition of saccharides resulted in weak gels and in some cases no gel was formed at all after one freeze and thaw step. The polyalcohols gave better results; the gels formed had approximately the same or a higher gel strength than in the absence of the cryoprotectant.

Immobilization experiments with adonitol as a cryoprotectant were done at several freezing and thawing rates, because adonitol protected the suspended cells relatively well and strong gels were formed. However, no significant growth of immobilized *Nitrosomonas europaea* was found. Therefore, we concluded that immobilization of *Nitrosomonas europaea* in PVA by iterative freezing and thawing is not possible. In contrast, growth of nitrifying bacteria immobilized in PVA with iterative freezing and thawing is reported by Willke *et al.* (1996). They used a mixed culture which was cultured in their laboratory for several years in such a way that the cells were relatively insensitive to changes in process conditions, for example low pH or low temperatures. This may explain the difference in growth and survival of the cells after immobilization with iterative freezing and thawing. Successful immobilization with this method was also found with other micro-organisms, for example *E.coli* (Ariga *et al.*, 1987), acclimated sludge (Myoga *et al.*, 1991) or *Z. mobilis* (Lozinsky *et al.*, 1996).

Growth of *Nitrosomonas europaea* in PEO was determined too. First the effect of the prepolymers, the initiator and the polymerization products on the activity of the cells was determined. No effect of the prepolymers and the initiator on the activity was found. The reaction products, however, decreased the activity slightly (a 10-20% decrease). After this, an immobilization experiment was done and the activity of the cells followed in time (see Figure 2).

The start-up phase was long. From the activity measurements (before and after immobilization) it was estimated that approximately 0.5% of the cells survived the immobilization procedure. This is a low value. Tanaka *et al.* (1996) reported similar low survival percentages between 0.2 and 2.1% for nitrifying sludge immobilized in polyethylene glycol (PEG). Presently, we are studying the possibilities for optimization of the immobilization in PEO in cooperation with HBG (Enschede, the Netherlands).

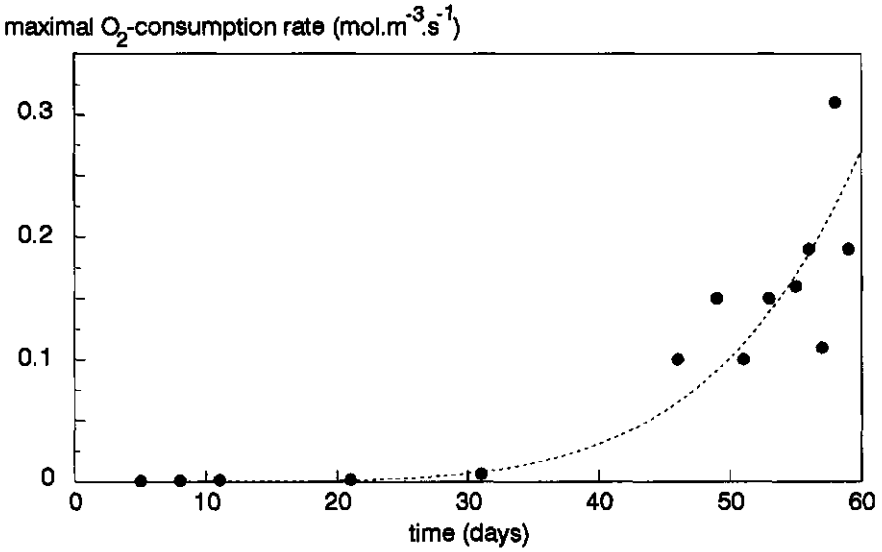

Figure 2. The activity (maximal oxygen consumption rate) of *Nitrosomonas europaea* cells immobilized in PEO.

Growth in PCS was observed earlier (Leenen *et al.*, 1996); cells survived the pH-shock and were able to grow in the support, but also here the start-up phase was long. The survival rate was determined to be approximately 0.9%.

Stability

In previous research (Leenen *et al.*, 1996) selection criteria for support materials for wastewater-treatment systems were set up. For this, several characteristics of natural and synthetic supports were determined and compared. It was concluded that polyvinyl alcohol (PVA), polycarbamoyl sulphonate (PCS) and polyethylene glycol (PEG), are promising materials for application in wastewater-treatment systems. They could not be solubilized in domestic wastewater, showed good mechanical properties and are reported to be not biodegradable.

In bioreactors, gel beads are continuously subjected to hydrodynamic shear stresses, bursting of gas bubbles, collisions with other gel beads and reactor parts. These forces can result in severe abrasion of the gel beads. The sensitivity of the beads to abrasion largely depends on their rheological properties. The relevance of these properties for the mechanical stability and their resistance to abrasion was studied by Martins dos Santos *et al.* (1996). They found that the resistance of gel materials to compression clearly decreased with the number of oscillations (simulated collisions) and that abrasion of polymer beads is likely to be the result of fatigue of the gel materials. Therefore, determining the compressive strength of a freshly prepared support does not give enough relevant information on the sensitivity to abrasion in a reactor. For this, repetitive compressions have to be done to determine how the rheological properties of the support changes during these compressions. If the properties alter only slightly the support is less sensitive to abrasion than if these properties change considerably (Martins dos Santos *et al.*, 1996).

The strain and stress at fracture of PCS, PEG and PVA were determined earlier (Leenen *et al.*, 1996; Martins dos Santos *et al.*, 1996). A new interesting support material is a chemically crosslinked polyethylene oxide (PEO), a polyethylene-glycol-like gel material developed by the Holland Biomaterials Group (Enschede, the Netherlands). This gel was studied in more detail in the research reported here.

The periodic application of stresses in a bioreactor is simulated by cyclic compression of a gel material (oscillation tests). The method as described by Martins dos Santos *et al.* (1996) was used. The evolution of the resistance to compression at 75% of their maximum compression is shown for two samples in Figure 3.

A decrease in the resistance to compression is related to microfracture and crack propagation in the gel. For each material used, these alterations will be different. 'Elastic' gels like PEG and PCS will accommodate the stresses better than more 'stiff' gels like alginate and carrageenan. Martins dos Santos *et al.* (1996) showed that alginate and carrageenan gels indeed changed fast during the first 100 compressions and that they abraded in bubble columns. In Figure 3 it can be seen that the resistance to compression of PEO decreased only a little with the number of oscillations, indicating that during this experiment the characteristics of the gel alter only minimally. PEO is therefore likely to be insensitive to abrasion.

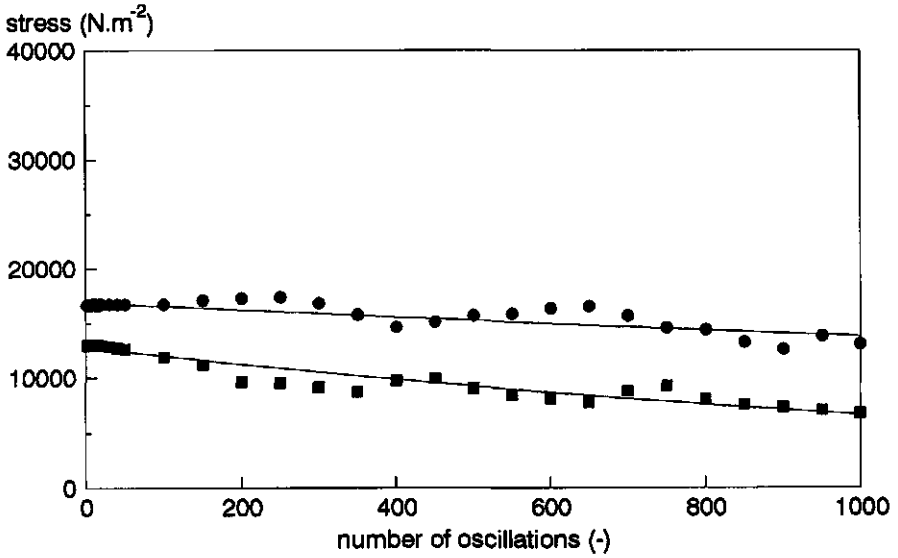

Figure 3. The evolution of the resistance to compression of PEO at 75% of maximum compression (in duplo).

Nitrification in wastewater

Both PCS and PEO are promising materials for application in wastewater: growth of nitrifying cells was obtained and the gels are likely to be stable. Presently, optimization of the immobilization procedure in PEO is being studied further in corporation with HBG. To study the possibility of immobilized nitrifying cells in domestic-wastewater treatment PCS was therefore chosen. A relatively low (7% w/v) gel load in the reactor was used, as only a limited amount of PCS was available.

An internal air-lift loop reactor (ALR) with nitrifying microorganisms immobilized in PCS was fed with pretreated domestic wastewater. This wastewater contained approximately 3 mole $NH_4^+.m^{-3}$ and 70 g $COD.m^{-3}$. Initially the reactor was fed with mineral medium with a gradual transfer to pretreated wastewater to have a slow decrease in ammonia concentration. After four days the reactor was fed with 100% pretreated wastewater. Every day the NH_4^+, NO_2^- and NO_3^- concentrations were analyzed. In Figure 4 the results of the period in pretreated wastewater are shown.

Figure 4 shows that nitrification occurred in this reactor. NH_4^+ removal took place and approximately 1-2 mole.m^{-3} was converted to NO_2^- and NO_3^- by the immobilized nitrifiers. In average 37% of the NH_4^+ was removed from the influent. The NH_4^+ removal in this reactor system showed much variation in time, which is partly due to the natural variations in the NH_4^+ concentration in the influent and partly due to the influent supply. At day 21, for example, the wastewater-treatment plant was malfunctioning and therefore the reactor could not be fed with fresh pretreated wastewater temporarily.

From these results it is clear that it is possible to have nitrification in an immobilized-cell system. This experiment was done at a short hydraulic retention time of about 20 minutes and a low gel load of 7% (v/v). Under these circumstances a nitrification capacity between 1 and 3 kg $N.m^{-3}_{reactor}.d^{-1}$ (between 13 and 40 kg $N.m^{-3}_{gel}.d^{-1}$) was accomplished. Increasing the hydraulic retention time and the gel load will lead to higher conversions. A gel load of, for example, 15% (v/v) may double the conversion reached.

In order to be able to compare the capacities reached, in this study, with those reached in other processes, the capacities were calculated to the surface area of the beads ($kg_N.m^{-2}_{gel}.d^{-1}$) as the experiments were not done at optimal conditions (e.g. gel load). The capacities reached are then between 4.8 - 14.5 $g_N.m^{-2}_{gel}.d^{-1}$.

In previous research in mineral media with a higher ammonium concentration (20 mM), 15-25% (w/v) gel load and a longer residence time (5-6 hours) capacities of approximately 5-7 $g_N.m^{-2}_{gel}.d^{-1}$ were reached (3 $kg_N.m^{-3}.d^{-1}$; Wijffels *et al.*, 1991). Tijhuis (1994) reached capacities around 4.5-5 $g_N.m^{-2}_{gel}.d^{-1}$ (5 $kg_N.m^{-3}.d^{-1}$) in the biofilm airlift suspension reactor at a liquid retention time of 1-2 h with a 95% reduction in the ammonium concentration in mineral medium. Sumino *et al.* (1992) were able to remove 0.07 $g_N.m^{-2}_{gel}.d^{-1}$ (0.2 $kg_N.m^{-3}.d^{-1}$) with nitrifying sludge immobilized in urethane. These capacities are all in the same range as found in this study, except for the latter.

The system investigated in this study has thus potential for usage in the treatment of domestic wastewater and can compete with conventional systems. Nitrification was found, the PCS gel beads did not dissolve in the pretreated wastewater and no abrasion of the beads was observed.

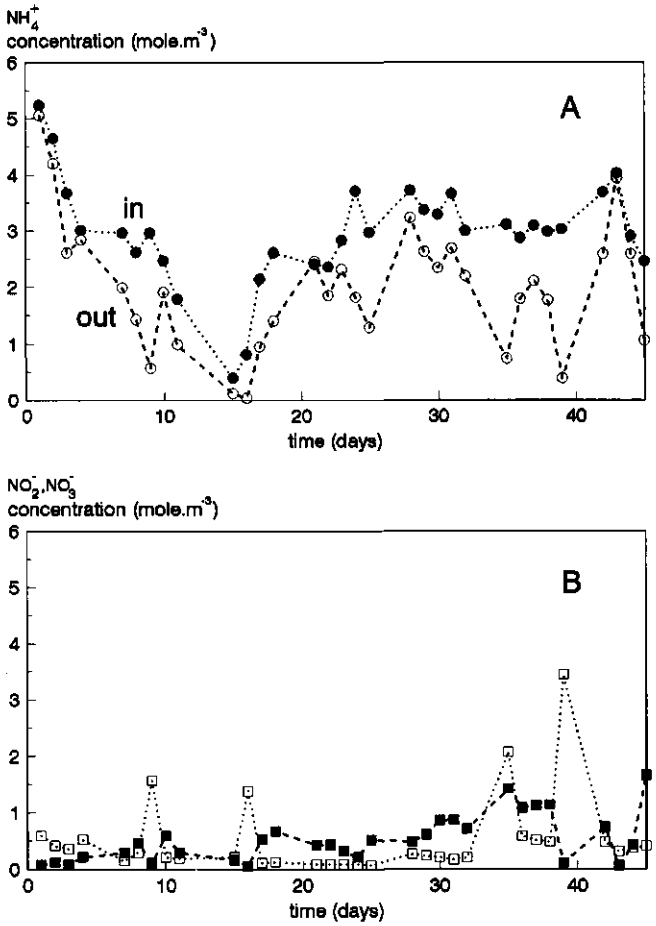

Figure 4. a) Concentration of ammonia in influent (●) and effluent (○).
b) Concentrations of nitrite and nitrate in effluent (■ NO_2^-, □ NO_3^-).

Heterotrophs

The influent and effluent COD-concentrations were analyzed daily to examine the activity of heterotrophic organisms (see Figure 5). No COD-removal in the reactor took place during the experiment.

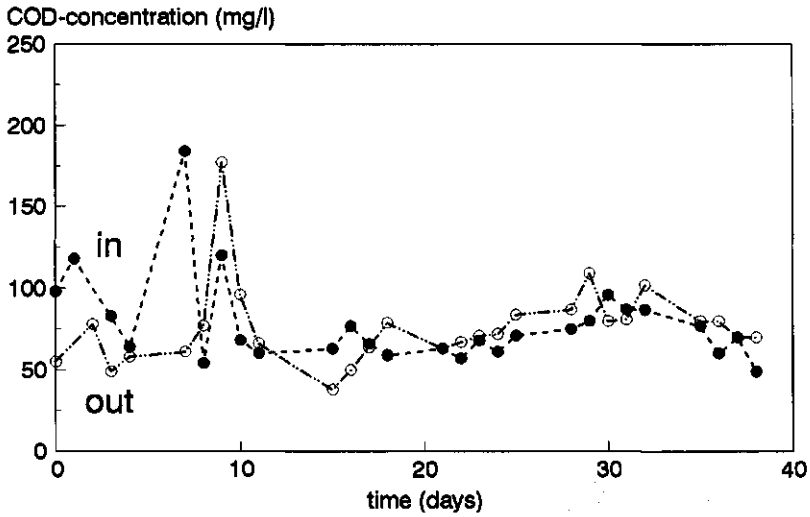

Figure 5. COD concentrations in influent (●) and effluent (○).

The presence of heterotrophic organisms on the gel beads was further examined by an activity test with acetate as the substrate. No acetate conversion was observed. From these results it can be concluded that heterotrophic organisms did neither attach to the gel beads nor were growing in the reactor.

Microscopic analyses of the gel beads, after 3 weeks of operation, showed a film formed on the gel beads. The thickness of this film was estimated to be around $2*10^{-3}$ m (see Figure 6). No COD-removal took place so the question arose what this could be? It appeared that this film consisted of attached ciliates (see Figure 7), probably of the genus *Vorticella* or *Carchesium* (Curds, 1969) and a few nematodes. These ciliates use bacteria as their sole energy source. These bacteria can either be the suspended bacteria which were present in the influent of the reactor or the nitrifying bacteria which were expelled from the PCS gel bead. In previous experiments and during the start-up phase with mineral medium attachment or occurrence of ciliates was never observed. Furthermore, after the experiment with pretreated wastewater was terminated, when the reactor was fed with mineral medium again, the film of ciliates disappeared. The ciliates therefore most likely fed themselves with the suspended biomass present in the influent flow.

The presence of these ciliates can be useful, because they catch suspended cells from the influent (Curds, 1975). A disadvantage of their presence can be that they compete with the nitrifying bacteria for oxygen. After 44 days of operation the nitrification activity of gel beads with a film was measured and compared with the activity of gel beads from which the film was removed. The nitrification activity of gel beads with ciliates was twice the activity of the beads without a film (respectively $4.1 * 10^{-3}$ and $1.9 * 10^{-3}$ mole $O_2.s^{-1}.m^{-3}_{gel}$). The presence of a film with ciliates thus did not hamper the nitrification capacity. Microscopic analyses showed that the film changed during the experiment. Initially the film consisted of merely ciliates, but later on biomass was attached to the stems of the ciliates (Figure 8). In addition to this the film had changed in colour from white to red. *Nitrosomonas europaea* is a red micro-organism and probably the cells attached to the stems of ciliates after they were expelled from the gel bead. Furthermore, the combination of diffusion and convection in this 'hairy' structure may increase the substrate availability.

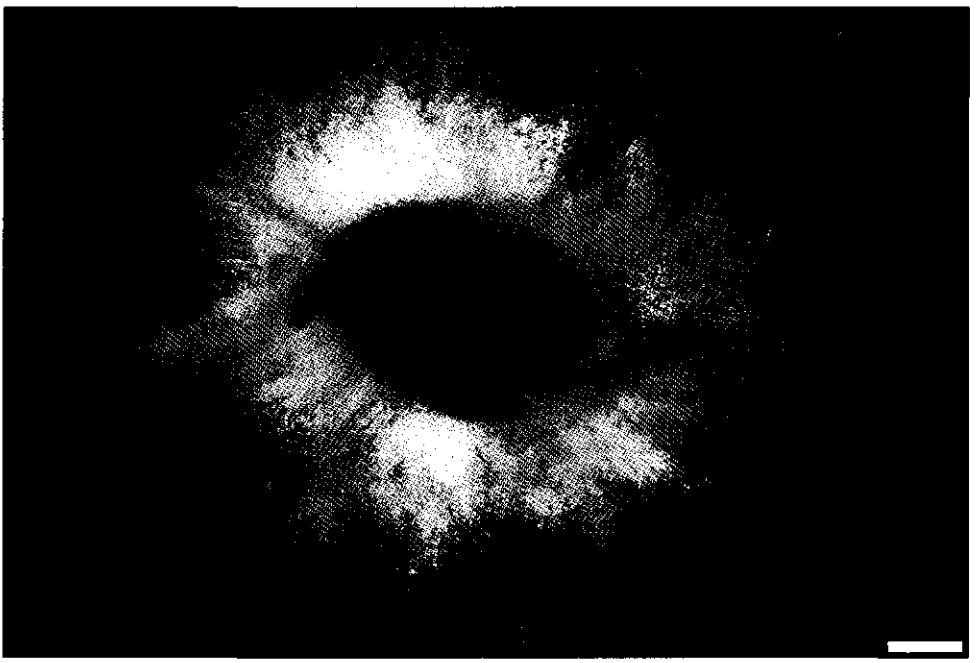

Figure 6. A PCS gel bead with immobilized nitrifying microorganism and attached film (bar length 0.5 mm).

Figure 7. An enlargement of a part of the attached ciliates film (bar length 25 μm).

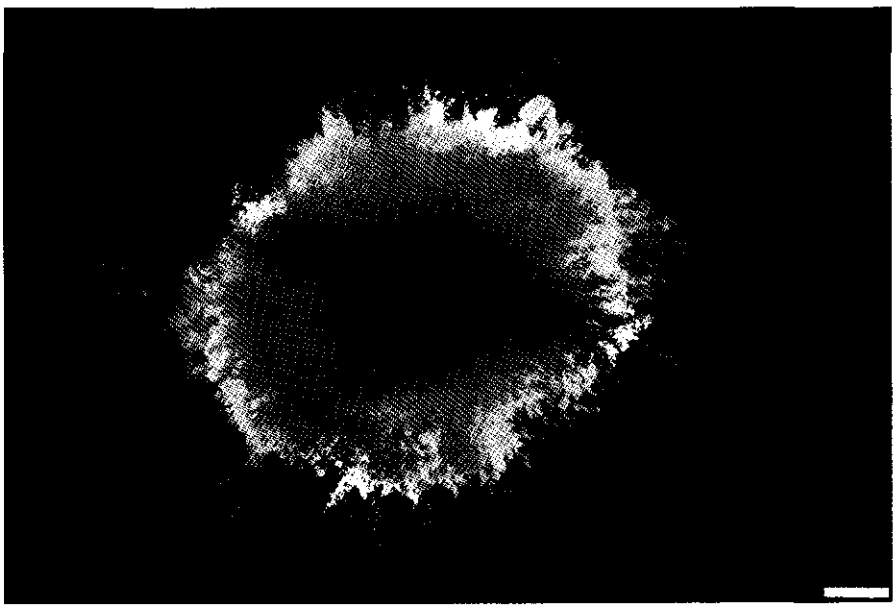

Figure 8. A PCS gel bead with immobilized nitrifying bacteria and attached film of ciliates and micro-organisms (bar length 0.5 mm).

CONCLUSIONS

Growth of *Nitrosomonas europaea* was obtained in PEO and PCS. In PVA, however, no significant growth was obtained, even in the presence of cryoprotectants.

PEO was found to be relatively insensitive to cyclic compressions, indicating that this gel material is relatively insensitive to abrasion.

It is possible to have nitrification in pretreated domestic wastewater with the aid of nitrifying microorganisms artificially immobilized in PCS.

No growth of heterotrophic bacteria was found, but a film of ciliates was observed on the bead surface. These attached ciliates did not hamper the nitrification activity, which is probably the result of the attachment of nitrifying bacteria to the stems of the ciliates.

ACKNOWLEDGEMENT

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CHAPTER 7

NITRIFICATION BY ARTIFICIALLY IMMOBILIZED CELLS
Model and practical system !

ABSTRACT

The feasibility of using immobilized nitrifying cells in domestic-wastewater treatment is discussed. For this, the design of an aeration tank of an activated-sludge process is compared with the design of an aeration tank for an immobilized-cell process. Especially, the effects of lower temperatures (7°C) and fluctuations in influent load (dynamics), as well as the choice of an appropriate support material are discussed.

The immobilized-cell process has promising characteristics to meet the desired demands for future wastewater-treatment plants, i.e. high biomass concentration, small volume of the aeration tank, low liquid retention times, reduced sludge production, and a high operational flexibility. Furthermore, the cost for production of immobilized cells are more than compensated for by the reduced costs for construction of a smaller reactor.

Nitrification with artificially immobilized cells: model and practical system !
E.J.T.M. Leenen, J. Tramper and R.H. Wijffels, submitted.

INTRODUCTION

Immobilized microbial cells are used in a variety of studies and industrial applications, e.g. for the production of beverages, such as beer (Van Iersel *et al.*, 1995) and sparkling wine (Gòdia *et al.*, 1991), pharmaceuticals (e.g. amino acids, Chibata *et al.*, 1992) and chemical intermediates (e.g. 7-amino-cephalosporamic acid, Tosa and Shibatani, 1995; diltiazan intermediate, Matumae *et al.*, 1994). Several studies have demonstrated that under numerous conditions immobilized cells have advantages over free cells, for example decreased temperature sensitivity (Leenen *et al.*, 1994; Wijffels and Tramper, 1995), protection from toxic substances (Barros *et al.*, 1987; Keweloh *et al.*, 1989) and sometimes increased metabolic activity and productivity (Gadkari, 1990). The recent increase in awareness of environmental problems has stimulated research on the use of immobilized cells as a new technology for environmental applications (Table I). So far, however, this research has been done on a small scale only and to our knowledge only the nitrification process with cells entrapped in polyethylene glycol (the PEGASUS process; Emori *et al.*, 1996; Takeshima *et al.*, 1993) has been realized on full scale.

In this paper the state-of-the-art and the possibilities for the application of entrapped nitrifying microorganisms for treatment of domestic wastewater are discussed.

FUTURE DEMANDS FOR WASTEWATER TREATMENT

In general, wastewater-treatment plants are based on the activated-sludge process. In this process wastewater is supplied to an aerated reactor containing suspended microorganisms, which remove and degrade the pollutants. In the settling tank the aggregated bacteria are separated from the water and partly recycled to the reactor. Due to the open structure of the aggregates the settling velocity is low and the sludge contains a high concentration of water. Therefore, only a low concentration of viable biomass can be recycled, resulting in a relatively low biomass concentration in the aeration tank.

Table I: Potential environmental applications of entrapped immobilized cells

Process	Species immobilized	Support material	Reference
nitrification	*N. europaea*, *N. agilis*	carrageenan, alginate	Wijffels *et al.*, 1991; Van Ginkel *et al.*, 1983; Leenen *et al.*, 1994; Lewandowski *et al.*, 1987
		(modified) polyurethane	Leenen *et al.*, 1994; Uemoto and Saiki, 1996
	nitrifying sludge/ mixed culture	polyethylene glycol (PEG)	Tanaka *et al.*, 1991; Sumino *et al.*, 1992[b]; Chudoba *et al.*, 1996; Emori *et al.*, 1996; Takeshima *et al.*, 1993; Roston *et al.*, 1996
		polyvinyl alcohol (PVA)	Roston *et al.*, 1996; Asano *et al.*, 1992; Chen and Lin, 1994; Hashimoto and Furukawa, 1987; Myoga *et al.*, 1991; Willke and Vorlop, 1996
		(modified) polyurethane	Roston *et al.*, 1996; Sumino *et al.*, 1992[a]; Willke *et al.*, 1994
	(micro)algae	carrageenan	Chevalier and de la Noue, 1991
nitrification and phosphorous removal	*Anabaena* CH3	alginate	Lee *et al.*, 1996[a]
nitrification and denitrification	*N. europaea* and *P. denitrificans*	carrageenan	Dos Santos *et al.*, 1992
nitrate removal	*Chlamydomonas reinhardtii*	alginate	Garbayo *et al.*, 1996
	denitrifying bacterium	carrageenan	Wijffels *et al.*, 1991
COD removal	sludge	cellulose-triacetate, alginate	Yang *et al.*, 1988
phenol removal	sludge	polyacrylamide	Ozaki *et al.*, 1991
		cellulose triacetate	Yang and Wang, 1990
removal of heavy metals	*Anabaena* CH3	alginate	Lee *et al.*, 1996[a]
removal of chlorinated compounds	*Pseudomonas* sp., *A. radiobacter*	alginate	Lee *et al.*, 1996[b]

The removal of ammonia (nitrification) or xenobiotics is accomplished by slow-growing microorganisms; the hydraulic retention time must therefore be long in order to keep these microorganisms in the reactor. Generally this is realized by increasing the reactor volume, resulting in a relatively low productivity. By using

immobilized cells the liquid retention time can be chosen independently from the biomass retention time. Therefore, higher volumetric capacities can be reached and smaller reactors can thus be used (Hunik *et al.*, 1994[a]; Wijffels *et al.*, 1991).

Table II: Future demands for wastewater-treatment plants in comparison with the activated-sludge and the immobilized-cell process.

	Activated-sludge process	Immobilized-cell process	Desired
mainly removal of	organic pollutants, nitrogen and phosphorous compounds	organic pollutants, nitrogen and phosphorous compounds, xenobiotics	all pollutants
volume of aeration tank [m^3.(1000 p.e.)$^{-1}$][a]	100-500[h]	2-6[b]	as small as possible
Biomass concentration ($g.l^{-1}$)	1-6	1-60[b,c]	as high as possible
Liquid retention time in aeration tank (h)	4-10	0.5-4[b,d]	as low as possible
Sludge production (kg/kg NH_4^+-N)	0.2[e]	0.05-0.10[b,g]	0
operational flexibility	low	high	high
energy requirement ($kWh.kg^{-1}$ O_2)	0.7-1.5[e]	0.5-1.3[f]	as low as possible

[a] p.e.= population equivalent
[b] calculated with the dynamic models of Hunik *et al.*, 1994[b] and Leenen *et al.*, 1996[a].
[c] Heijnen and Van Loosdrecht, 1990
[d] Takeshima *et al.*, 1993; Sumino *et al.*, 1992[a]
[e] Tchobanoglous, 1979
[f] Van 't Riet and Tramper, 1991
[g] Tijhuis *et al.*, 1994
[h] mean value of several activated-sludge plants

In Table II the desired future demands for wastewater-treatment plants are given and compared with the characteristics of the activated-sludge and the immobilized-

cell processes. Microorganisms degrading or accumulating problematic compounds (e.g. xenobiotics, heavy metals) can easily be introduced into an immobilized-cell reactor, without the risk of excessive biomass loss, and in this way the desired removal demands can be met. Immobilized-cell reactors can be rather small mainly due to the high biomass concentration reached in these systems, resulting in low liquid retention times. According to calculations with dynamic models (Hunik *et al.*, 1994[b], Leenen *et al.*, 1996[a]) the volume of an immobilized-cell reactor can be approximately 100 times smaller than the aeration tank in a conventional activated-sludge plant. Additionally, the sludge production per kg NH_4^+-N consumed in an immobilized-cell reactor was calculated (Hunik *et al.*, 1994[b], Leenen *et al.*, 1996[a]) to be approximately half the amount of the sludge produced in an activated sludge plant. This is caused by the surplus of biomass in the centre of the bead, which only consumes some substrate to satisfy the maintenance requirements.

In the activated-sludge plant the aeration tanks are shallow (low and wide geometry), a reactor geometry which is rather unfavourable for oxygen transfer (per unit energy). In industry, optimized stirred-tank reactors with a low energy utilization (0.4 - 0.5 $kWh.kg^{-1}$ O_2; Heijnen and Van Loosdrecht, 1990) are used and future treatment plants must be designed in such a way that the energy requirements are minimal. The immobilized-cell reactors developed are mainly air-lift reactors or bubble columns which have a oxygen transfer. The energy utilization is, however, higher than in stirred-tank reactors, because more energy is needed to inject the oxygen into the vessel. Another possible advantage of immobilized-cell processes is that generally a reactor with a small surface area is used, which is easy to cover to decrease the emissions of odour, noise and aerosols.

Immobilized cells are investigated in great detail and are well described by dynamic models. These models can be used to design the overall process and for determining the parameters to be measured and controlled. Therefore, also the operational flexibility of these reactors is high (see dynamics part below).
In conclusion, immobilized-cell reactors can be an interesting option to meet the desired future demands for treatment of domestic wastewater.
In this study, nitrification with artificially immobilized cells is used as an example of a possible application of an immobilized-cell process.

NITRIFICATION WITH ARTIFICIAL IMMOBILIZED CELLS

Nitrification with entrapped cells has been studied for several years (Kokufuta *et al.*, 1982; Lewandowski *et al.*, 1987; Tada *et al.*, 1990; Tramper and de Man, 1986; Van Ginkel *et al.*, 1983; Wijffels and Tramper, 1995). A schematic representation of the system used in our group is shown in Figure 1. This immobilized-cell system was mainly used as a model to study the growth of the entrapped biomass, the substrate transport and their interactions. Model studies have shown that indeed high capacities can be reached; this has been the reason to study the practical applicability as well.

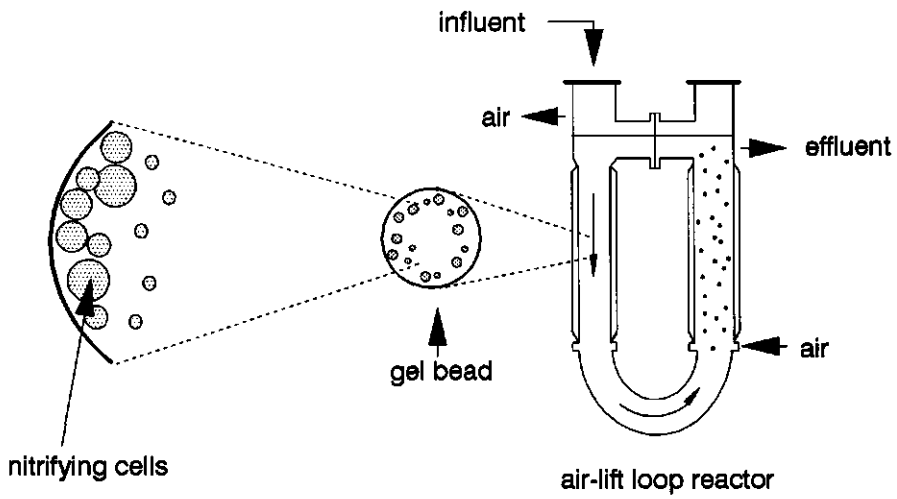

Figure 1. Schematic representation of an immobilized-cell process for nitrification.

Mostly, experiments are done at optimum conditions or at defined steady-state situations and relatively little information is gathered until now about the dynamics of the process at fluctuating conditions (e.g. fluctuations in substrate load or temperature). This lack of knowledge together with the unknown physical and chemical stability of the support hampers the application of immobilized cells. The feasibility of immobilized nitrifying cells in domestic-wastewater treatment is discussed in this study.

DYNAMICS

In wastewater-treatment plants the process conditions are continuously changing. Treatment systems need to be designed in such a way that fluctuations in for example water temperature and substrate loadings have no significant negative effect on the effluent quality. The capacity and rate of immobilized-cell processes are determined by the combination of the biochemical-reaction rate (kinetics of the entrapped biomass) and the rate of diffusion of substrates to and in the gel bead. Generally, in immobilized-cell reactors a high biomass concentration is present (see Figure 2), but due to diffusional restrictions only the biomass near the periphery of the gel bead is active (Stormo and Crawford, 1992; Wijffels *et al.*, 1991; Willaert, 1996).

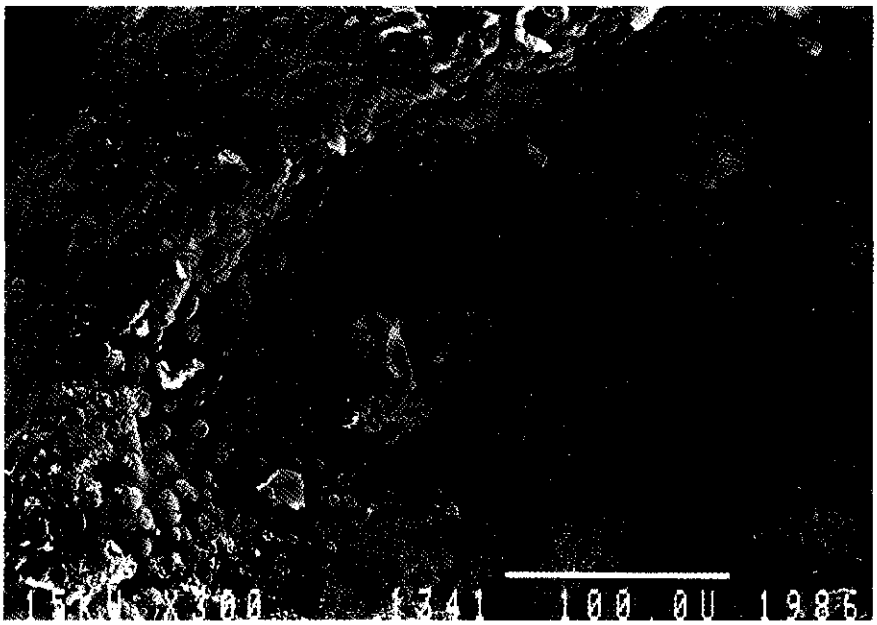

Figure 2. A carrageenen gel bead with micro-colonies of *Nitrosomonas europaea*. (bar length 100 μm).

The design of a nitrification tank based on either immobilized cells or activated sludge (with and without 50% sludge recycle), is shown in the Text box. As standard situation an average capacity of $2x10^4$ $m^3.d^{-1}$ with an influent concentration of 3.5 mole $NH_4^+.m^{-3}$ at 30°C was chosen.

Text box: Design of an aeration tank for nitrification in the activated-sludge and the immobilized-cell process.

Design criteria: average capacity: $2x10^4$ $m^3.d^{-1}$
NH_4^+ influent: 3.5 $mole.m^{-3}$ (= 59 $mg.l^{-1}$)
average temperature: 30°C
peak capacity: $6x10^4$ $m^3.d^{-1}$
peak NH_4^+ influent: 6 $mole.m^{-3}$
NH_4^+ effluent: 0.5 $mole.m^{-3}$

	Activated sludge*		Immobilized cells#	
	30°C	7°C	30°C	7°C
NH_4^+ effluent ($mole.m^{-3}$)	0.5	0.5	0.5	0.5
liquid retention time (h)	42	351	0.75	0.9
Volume (m^3)	35000	293000	625	750
	with 50% sludge			
NH_4^+ effluent ($mole.m^{-3}$)	0.5	0.5		
liquid retention time (h)	28	231		
Volume (m^3)	23800	195000		
	peak capacity	peak NH_4^+	peak capacity	peak NH_4^+
NH_4^+ influent ($mole.m^{-3}$)	3.5	6	3.5	6
NH_4^+ effluent ($mole.m^{-3}$)	0.5	0.5	0.5	0.5
liquid retention time (h)	42	84	0.75	1
Volume (m^{-3})	$10.5x10^4$	$7x10^4$	1250	833
	with 50% sludge recycle			
NH_4^+ effluent ($mole.m^{-3}$)	0.5	0.5		
liquid retention time (h)	28	55		
Volume (m^{-3})	$7x10^4$	$4.5x10^4$		

Activated-sludge system: Biomass concentration: 4000 $g.m^{-3}$
Percentage nitrifiers: 5% (= 200 $g.m^{-3}$)
NH_4^+ oxidation rate: $8.6x10^{-3}$ $mole.g^{-1}.d^{-1}$ (= 120 mg $NH_4^+.g^{-1}_{nitrifiers}.d^{-1}$)

Immobilized-cell system: Initial concentration nitrifiers: $4x10^{-3}$ $kg.m^{-3}_{gel}$ = $1x10^{-3}$ $kg.m^{-3}_{reactor}$
Concentration nitrifiers at end: 58 $kg.m^{-3}_{gel}$ = 14.5 $kg.m^{-3}_{reactor}$

* calculated according to Barnes and Bliss, 1983.
\# calculated with the dynamic models of Hunik *et al.*, 1994[b] and Leenen *et al.*, 1996[a].

In the upper part of the Text box the effect of a low temperature (7°C) is calculated. If the temperature in an activated-sludge plant is decreased from 30°C to 7°C the volume of the aeration tank has to be increased by a factor 8. If this temperature shift is occurring in an immobilized-cell reactor the volume only has to be increased slightly (1.2 times). This is caused by the surplus of biomass in the centre of the bead, acting as an intrinsic safety factor in the design of immobilized-cell processes. The process is thus not limited by the activity of the immobilized cells, but by diffusion of the substrate. The latter is less temperature sensitive than the activity of the cells. The immobilized cells are indeed, as was shown previously (Hunik *et al.*, 1994[a]; Leenen *et al.*, 1994; Wijffels *et al.*, 1995), less sensitive to low temperatures than suspended cells because of this diffusion limitation.

In the lower part of the Text box the effect of peak loading (peak capacity or peak NH_4^+) on the design of an aeration tank is calculated. At a peak capacity of $6x10^4$ $m^3.d^{-1}$ (3 times higher than average capacity) the aeration tank of an activated-sludge process (with and without sludge recycle) has to be increased accordingly (3x larger tank volume). The volume of the aeration tank of an immobilized-cell process, however, has to be increased only twice with the same liquid retention time as at average capacity. If the influent ammonium concentration increases and the effluent demand is the same (0.5 mole $NH_4^+.m^{-3}$) the volume of the aeration tank in the activated-sludge process and the liquid retention time have to be doubled. In the immobilized-cell process, however, both the volume of the aeration tank as the liquid retention time have to be increased only slightly (1.33 times). The immobilized-cell process is less sensitive to peak loadings as a result of the surplus of biomass in the centre of the bead, acting as an intrinsic safety factor.

If fluctuations in substrate loadings occur regularly, the immobilized-cell process will adapt almost instantaneously to the new situation (see Figure 3a). The overall capacity and effluent ammonium concentration will hardly change. If these fluctuations, however, do not occur regularly, but only a few times a year, the system will adapt slower to the new situations (see Figure 3b). This is caused by the decay of biomass in the centre of the bead (Leenen *et al.*, 1996[a]). The death rate of biomass is higher when ammonium is the limiting substrate than when oxygen is limiting. Therefore, if the influent ammonium concentration is low it is necessary to decrease the oxygen input too, in order to decrease the rate of

biomass decay (Leenen *et al.*, 1996[a]).

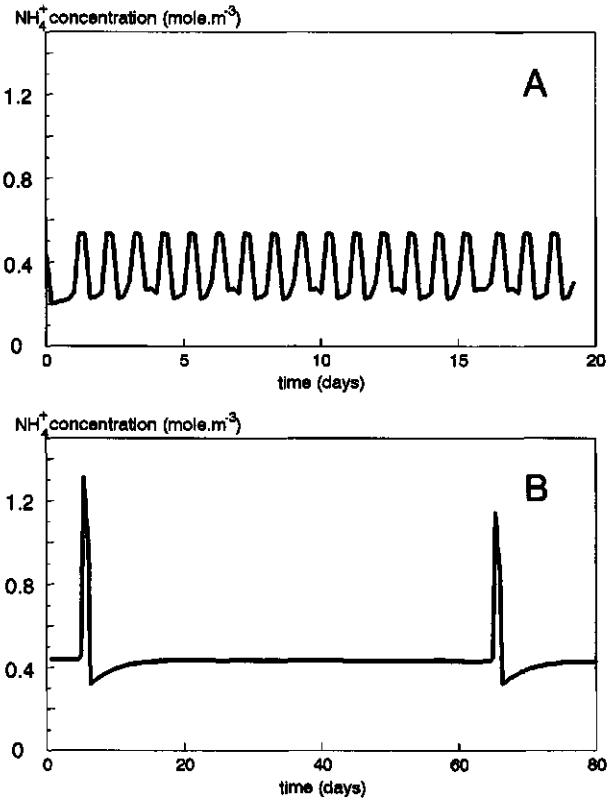

Figure 3. Effect of fluctuating influent ammonium concentration on the effluent ammonium concentration.
a) daily fluctuations, b) fluctuations every 2 months
(liquid retention time= 1 hour, average influent concentration= 3.5 mole.m^{-3}, peak concentration= 6 mole. m^{-3}).

In conclusion, immobilized-cell processes are less sensitive to changes in temperature and substrate loading than activated-sludge systems. In fact, immobilized cells are capable to have almost constant capacities at fluctuating conditions.

The dynamics of the immobilized-cell system have been described well in several mathematical models (De Gooijer *et al.*, 1991; Hunik *et al.*, 1994[b]; Leenen *et al.*, 1994; Monbouquette *et al.*, 1990; Wijffels *et al.*, 1996; Willaert, 1996). These models can form an important tool for the design of wastewater-treatment plants with immobilized cells (see Table II and Text box; Hunik *et al.*, 1994[a]). These models also allow the application of on-line dynamic process control leading to process optimization (De Backer *et al.*, 1996).

SUPPORTS

Most studies on immobilized cells are done with natural support materials, like alginate and carrageenan. However, these supports dissolve in water if their counterions are not present such as is the case in domestic wastewater, are biodegradable and suffer from abrasion (Leenen *et al.*, 1996[b]). For application of immobilized cells for the treatment of wastewater a support material with the following characteristics is needed:

* insoluble,
* not biodegradable,
* not sensitive to abrasion,
* high diffusion coefficients for substrates, and
* a relative simple immobilization procedure with a high retention of viable biomass.

The mechanical stability (sensitivity to abrasion) of the support is of crucial importance for application of immobilized-cell technology in bioconversions (e.g. wastewater treatment). The common methods for evaluation of the 'strength' of polymer beads (mostly force-to-fracture or tensile tests) are not relevant for the assessment of the mechanical stability against abrasion in bioreactors. It was found by Martins dos Santos *et al.* (1996) that the resistance of gel materials to compression clearly decreased with the number of oscillations (simulated collisions) and that abrasion of polymer beads is likely to be the result of fatigue of the gel materials. Therefore, determining the compressive strength of a freshly prepared support does not provide enough relevant information on the sensitivity to abrasion in a reactor. For this, repetitive compression is necessary to determine the change in rheological properties of the support during these compressions. If

the properties alter slowly the support is less sensitive to abrasion than if these properties change quickly (Martins dos Santos *et al.*, 1996).

Several synthetic supports are reported in which growth of biomass is observed and good stability in domestic wastewater is found (e.g. polyvinyl alcohol, polycarbamoyl sulphonate, polyethylene glycol; Asano *et al.*, 1992; Emori *et al.*, 1996; Hashimoto and Furukawa, 1987; Muscat *et al.*, 1993; Myoga *et al.*, 1991; Sumino *et al.*, 1992[a,b]; Tanaka *et al.*, 1991; Vorlop *et al.*, 1992; Wu and Wisecarver, 1992). A new promising material is a chemically crosslinked polyethylene oxide from Holland Biomaterials Group (the Netherlands). This material could not be solubilized in domestic wastewater, is not biodegradable, not significantly affected by abrasion and nitrifying cells survived the immobilization procedure and were able to grow in it (unpublished results).

The synthetic supports mentioned are all promising for usage in treatment plants with immobilized cells and should be investigated further to optimize the immobilization procedure.

FEASIBILITY STUDY

At the start of this research a theoretical feasibility study was done by a firm of independent consulting engineers (Witteveen-Bos, 1991). As at that time only experiments in defined medium were done with nitrifying cells encapsulated in alginate or carrageenan, this model process was used to compare with existing treatment systems. For this, an existing conventional activated-sludge plant (100.000 population equivalent; 0.15 kg $BOD.kg^{-1}_{dry\ weight}.d^{-1}$) for which the nitrification capacity had to be increased was chosen. The nitrification capacity was theoretically increased by either doubling the aeration volume or by adding a nitrifying immobilized-cell process behind the settling tank or before the aeration tank. The total costs were compared. The immobilized-cell process investigated consisted of an air-lift loop reactor with nitrifying bacteria entrapped in alginate. Beads for inoculation or replacement were produced in a separate unit.

As basis for the design a capacity of 2 kg $N.m^{-3}_{reactor}.d^{-1}$ was used for a reactor designed behind the aeration tank and 1 kg $N.m^{-3}_{reactor}.d^{-1}$ if the reactor was placed before the aeration tank; the latter to account for the possible attachment of heterotrophic organisms on the bead surface, which may decrease the oxygen

availability for the entrapped nitrifiers and therefore decrease the nitrification capacity.

The total costs consisted of investment costs (construction, finance, sales tax, gel-bead preparation costs and sundries), running costs (employees, overheads, maintenance and energy costs), debit of electromechanical parts in 15 year, and debit of the civil engineering part in 30 year. An increase in energy costs of 9% per year and an inflation of 5% was taken into account. The duration of the project was set at 30 years. In the calculations two variants were used: replacement of the support material once in 2 months or 2 years. In Table III the relative savings, calculated in this theoretical study, are given in comparison to the activated-sludge system with an increased aeration volume.

Table III: The relative savings of several designs in comparison with an activated-sludge process with an increased aeration volume.

		relative savings (%)
Activated sludge with increased aeration volume		- reference
Immobilized cells after aeration tank	new support 1 x 2 months	+6
	new support 1 x 2 year	+12
Immobilized cells before aeration tank	new support 1 x 2 months	0
	new support 1 x 2 year	+8

The conclusion was: the cost for production of alginate beads are compensated by the reduced costs for construction of a smaller reactor if the support material remains stable for at least two months. If the immobilized-cells system is placed behind the conventional installation the relative savings are higher (Witteveen-Bos, 1991).

As this theoretical feasibility study was done at the start of this research the design was based on experimental results from studies in mineral medium. Presently, however, it is known that it is indeed possible to obtain nitrification in domestic wastewater and high capacities (up to 4 kg $N.m^{-3}d^{-1}$) can be reached, which may result in higher theoretical savings if an nitrifying immobilized-cell process would be used to increase the aeration volume of an existing activated-sludge plant.

The land prices were not incorporated. In countries with a high population density (e.g. the Netherlands) the land is expensive. Using a compact treatment process will therefore decrease the treatment costs.

In the feasibility study alginate was used as immobilization material. If a synthetic support is used with a much longer durability than alginate and approximately the same cost price as alginate, these savings will be even higher. In Table IV the estimated polymer costs of alginate pellets are compared with the costs of the chemically crosslinked polyethylene oxide pellets. As can be seen from this Table the price of the raw material of polyethylene oxide is lower than the costs of alginate. Some derivation of the polyethylene oxide, however, is needed and therefore the costs for production of 1 m^3 gel will be comparable with the cost for production of alginate gel. The polyethylene oxide gel, however, has better characteristics than alginate (no solubilization, not biodegradable and hardly sensitive to abrasion). The durability of this gel is therefore high, which increases the possible savings if this material is used in the treatment of domestic wastewater.

Table IV: The estimated costs of alginate and chemically crosslinked polyethylene oxide pellets.

	alginate*	polyethylene oxide#
price raw material (fl.kg^{-1})	20-50	10-25
price m^3 gel (fl)	600-1000	300-750

* 2-3% (w/v) alginate gel

\# 10% (w/w) crosslinked polyethylene oxide gel ---> gel swells approximately 3-4 times after crosslinking ---> 2-4% (w/v) polymer in swollen gel.

PERSPECTIVES FOR APPLICATIONS

In Table I possible applications of immobilized cells for wastewater treatment are given. Most of these studies are done at small scale with synthetic wastewater. In Japan, however, nitrification of domestic wastewater with cell immobilized in polyethylene pellets is already done at full scale. The reported volumetric capacity is low (about 0.25-0.3 kg $N.m^{-3}_{reactor}.d^{-1}$). This is caused by the relative low amount of pellets in the system and the low biomass concentration in

the pellets. By increasing the pellet concentration to 25% of the total reactor volume and by decreasing the liquid retention time, the volumetric capacity can be theoretically increased to 0.8-3 kg $N.m^{-1}_{reactor}.d^{-1}$. The used polyethylene glycol pellets show long durability and the nitrification capacity found is stable for several years now (Takeshima *et al.*, 1993).

A scale-up strategy for a nitrification process with immobilized cells was presented by Hunik *et al.* (1994[a]). The study was based on an air-lift loop reactor design (see Figure 4) and their dynamic models. A regime analysis was done to determine the rate-limiting step and as a result a set of design rules was obtained. This study can form a basis for further scale-up of immobilized-cell processes.

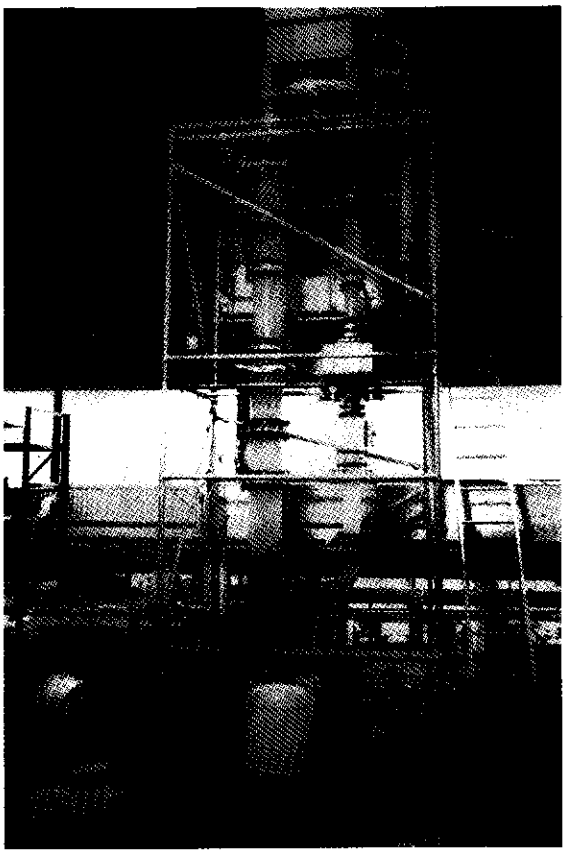

Figure 4. A pilot-scale air-lift loop reactor of 165 dm^3.

CONCLUSIONS

Immobilized-cell technology forms an interesting new development to meet the desired demands for future wastewater-treatment plants:

* a relatively small reactor can be used because of the high biomass concentrations and as a result low liquid retention times; therefore only a small part of the plant has to be occupied with the immobilized-cell reactor;
* microorganisms degrading or removing organic pollutants, nutrients or xenobiotics can easily be introduced into the system;
* a more stable reactor performance than the activated-sludge system can be accomplished even at low temperatures and fluctuating substrate loading;
* the costs for production of the support material are more than compensated by the reduced costs for construction of a smaller reactor.

By using the developed dynamic models the influence of several fluctuations can be investigated. These models can therefore form the basis of a design for a treatment plant and can be used to control the process on-line leading to process optimization. Some pilot studies in domestic wastewater are, however, necessary to validate and optimize the overall system.

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SUMMARY

The research described in this dissertation started after extended studies on the fundamental aspects of the growth of immobilized nitrifying cells. In these previous studies dynamic models were developed and experimentally validated, which resulted in knowledge about which steps determine the rate of the nitrification process and to which parameters the immobilized-cell process is sensitive. Furthermore, it showed that this system may be interesting as a practical system as well.

The aim of the work described in this thesis was to obtain more insight in the complex interactions in the nitrification process with artificially immobilized cells in air-lift loop reactors. The knowledge obtained may form the basis for a rational design of compact systems for practical applications, e.g. nitrification of domestic wastewater. This knowledge was built up by developing dynamic models, by studying the applicability and mechanical stability of the immobilization support and by doing experiments in domestic wastewater.

In Chapter 1 an overview of the previous studies and the approach, followed in our group for the development of a process with immobilized cells, are given. The subjects in this Chapter provide the starting points of the following chapters, which are arranged in three themes: dynamics, supports and application.

The first theme, the dynamics of immobilized nitrifying cells entrapped in carrageenan, is described in Chapter 2 and 3. The results in Chapter 2 clearly show the reduced temperature sensitivity of immobilized nitrifying cells caused by diffusion limitation. In Chapter 3 is described that, for example at low substrate concentrations and low hydraulic retention times, the death rate of cells is an important parameter. If cells die as a result of e.g. a low substrate loading, the process reacts slower to changes in substrate concentrations than if all cells remain viable.

The second theme (Chapter 4 and 5) deals with the selection and characterization of support materials for cell immobilization. In order to establish which support materials are the most suitable, characteristics of several natural and synthetic materials have been determined and compared. Natural gel materials, like alginate and carrageenan, have a mild immobilization procedure such that few cells die and they grow well in these supports. These supports, however, appeare to be soluble, biodegradable and sensitive to abrasion.

Synthetic gels, on the contrary, have better mechanical properties, but the immobilization conditions are less mild resulting in low biomass retention. For application of entrapped nitrifying cells synthetic gels, like polyethylene glycol, polyvinyl alcohol and polycarbamoyl sulphonate, are, however, promising (Chapter 4). In Chapter 5 the relevance of rheological properties of gel beads for their mechanical stability is described. It is concluded that tests based on rupture of 'virgin' beads does not provide relevant information on the mechanical stability and sensitivity to abrasion. It is likely that abrasion of gel beads is related to 'fatigue' of the gel materials.

Application of artificially immobilized nitrifying cells is the topic of the last theme, in which the two formerly discussed themes are combined. In the work described in Chapter 6 the applicability of promising support materials was studied in more detail and a suitable support was used to immobilize nitrifying cells. High nitrification capacities could be reached in pretreated domestic wastewater, demonstrating that it is indeed possible to apply this system. The possibilities, drawbacks and prospects of immobilized cells for environmental applications are discussed further in Chapter 7. It is demonstrated there on the basis of the gathered knowledge that the immobilized-cell process has promising characteristics to meet the desired demands for future wastewater-treatment plants i.e. high biomass concentration, small volume of aeration tank, low liquid retention times, reduced sludge production and a high operational flexibility. Furthermore, it was calculated that the costs for production of immobilized cells are more than compensated for by the reduced costs for construction of a smaller reactor.

SAMENVATTING

Het onderzoek beschreven in dit proefschrift is gestart na uitgebreid onderzoek aan de fundamentele aspecten van groei van geïmmobiliseerde nitrificerende micro-organismen. In deze voorgaande studies zijn dynamische modellen ontwikkeld en experimenteel gevalideerd. Dit heeft bijgedragen tot het verkrijgen van kennis over de snelheidsbepalende stappen in dit proces en voor welke parameters het proces met geïmmobiliseerde nitrificerende cellen gevoelig is. Tevens werd duidelijk dat dit proces ook als praktische toepassing interessant kan zijn.

Het doel van het werk beschreven in dit proefschrift was om het inzicht in de complexe relaties in het nitrificatieproces met geïmmobiliseerde nitrificerende cellen te vergroten. De vergaarde kennis kan de basis vormen voor het ontwerpen van compacte systemen voor praktische toepassingen, zoals b.v. de rioolwaterzuivering. Deze kennis werd verkregen door het ontwikkelen van dynamische wiskundige modellen, het bestuderen van de toepasbaarheid en de mechanische stabiliteit van dragermaterialen, en door het uitvoeren van experimenten in minerale media en voorbehandeld huishoudelijk afvalwater.

In hoofdstuk 1 wordt een overzicht van voorgaand onderzoek beschreven en tevens de in onze groep gevolgde aanpak om een proces met geïmmobiliseerde cellen te ontwikkelen gegeven. De onderwerpen in dit hoofdstuk zijn de uitgangspunten voor de volgende hoofdstukken, welke in 3 thema's zijn onderverdeeld, te weten: dynamiek, dragermaterialen en toepassing.

Het eerste thema, de dynamiek van cellen geïmmobiliseerd in carrageen, is beschreven in de hoofdstukken 2 en 3. De resultaten in hoofdstuk 2 laten duidelijk de ongevoeligheid van geïmmobiliseerde nitrificerende cellen voor temperatuurs-veranderingen zien als gevolg van diffusielimitatie. In hoofdstuk 3 wordt beschreven dat, bij bijvoorbeeld lage substraatconcentraties en lage vloeistofverblijftijden, de afsterfsnelheid van cellen van belang is. Indien cellen doodgaan door b.v. een lage substraatconcentratie zal het proces langzamer reageren op veranderingen in de substraatbelastingen dan indien alle cellen blijven leven.

Het tweede thema (hoofdstuk 4 en 5) behandelt de selectie en karakterisering van dragermaterialen voor immobilisatie van cellen. Om verschillende dragermaterialen te kunnen vergelijken zijn de karakteristieken van natuurlijke en gesynthetiseerde gelen bepaald. Natuurlijke gelen, zoals carrageen en alginaat,

maken een milde immobilisatiemethode mogelijk waardoor weinig cellen doodgaan en ze gemakkelijk in het gel kunnen vermenigvuldigen. Echter, deze materialen blijken oplosbaar, biologisch afbreekbaar en slijtagegevoelig. Synthetische gelen vertonen betere mechanische eigenschappen, maar de immobilisatieprocedures zijn niet mild, waardoor weinig biomassa de immobilisatie overleeft. Toch zijn synthetische dragermaterialen, zoals polyethyleenglycol, polyvinylalcohol en polycarbamoylsulfonaat, geschikt voor een toepassing van geïmmobiliseerde nitrificerende cellen (hoofdstuk 4). In hoofdstuk 5 wordt de relevantie van rheologische eigenschappen voor de mechanische stabiliteit beschreven. Geconcludeerd wordt dat het meten van de kracht (of weerstand) waarbij een pas gemaakt gel breekt of scheurt geen relevante informatie over de mechanische stabiliteit of de slijtagegevoeligheid van een gel geeft. Slijtage van een gel is afhankelijk van 'materiaalmoeheid' van het gelmateriaal.

Het toepassen van geïmmobiliseerde nitrificerende micro-organismen is het onderwerp van het laatste thema, waarin de beide voorgaande thema's worden gecombineerd. In het werk beschreven in hoofdstuk 6 werd de toepasbaarheid van veelbelovende dragermaterialen nader onderzocht en een geschikt dragermateriaal gebruikt voor immobilisatie van nitrificeerders. Hoge nitrificatiecapaciteiten werden bereikt in voorbehandeld huishoudelijk afvalwater, wat aangeeft dat dit proces inderdaad praktische toepassingsmogelijkheden heeft. De mogelijkheden, nadelen en verwachtingen voor het toepassen van dit systeem voor milieuvraagstukken wordt nader behandeld in hoofdstuk 7. Aangetoond wordt daar, op basis van de vergaarde kennis, dat het geïmmobiliseerde-celproces veelbelovend is en voldoet aan de eisen van toekomstige rioolwaterzuiveringsinrichtingen, n.l.: hoge biomassaconcentraties, klein volume van de beluchtingstank, lage vloeistofverblijftijden, verminderde slibproduktie en een hoge operationele flexibiliteit. Tevens werd berekend dat de produktiekosten van geïmmobiliseerde cellen ruim gecompenseerd worden door de lagere bouwkosten van een compacte reactor.

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Curriculum vitae

Imke Leenen werd op 19 augustus 1965 geboren in Blerick. In 1984 behaalde zij het VWO-diploma aan het college Marianum te Venlo. Zij vervolgde haar opleiding aan de Landbouwuniversiteit Wageningen. In 1990 studeerde zij af in de studierichting Levensmiddelentechnologie met als afstudeervakken Proceskunde en Industriële Microbiologie. Ze heeft stages gedaan bij het Central Food Technology Research Institute te Mysore (India) en bij Bioorganische Chemie van D.S.M. Research te Geleen.

Tot eind 1991 was zij wetenschappelijk medewerker bij de Landbouwuniversiteit bij de secties Proceskunde en Industriële Microbiologie. Vanaf 1 december 1991 is zij werkzaam geweest bij de sectie Proceskunde als assistent in opleiding en werkte zij aan het in dit proefschrift beschreven onderzoek.

Tot april 1997 is zij daarna werkzaam geweest bij dezelfde sectie als toegevoegd onderzoeker.