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Water Research 36 (2002) 469–481

WATER RESEARCH

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Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon

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Received 29 September 2000; received in revised form 09 January 2001; accepted 1 February 2001

Abstract

Two biofilm reactors operated with hydraulic retention times of 0.8 and 5.0 h were used to study the links between population dynamics and reactor operation performance during a shift in process operation from pure nitrification to combined nitrification and organic carbon removal. The ammonium and the organic carbon loads were identical for both reactors. The composition and dynamics of the microbial consortia were quantified by fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes combined with confocal laser scanning microscopy, and digital image analysis. In contrast to past research, after addition of acetate as organic carbon nitrification performance decreased more drastically in the reactor with longer hydraulic retention time. FISH analysis showed that this effect was caused by the unexpected formation of a heterotrophic microorganism layer on top of the nitrifying biofilm that limited nitrifiers oxygen supply. Our results demonstrate that extension of the hydraulic retention time might be insufficient to improve combined nitrification and organic carbon removal in biofilm reactors.

Keywords: Nitrification; Biofilm; Organic carbon; Hydraulic retention time; Fluorescence in situ hybridization

1. Introduction

The competition between heterotrophic and nitrifying bacteria for substrates (oxygen and ammonia) and space in biofilms is of major practical importance and thus has been the subject of several previous studies (for example [1–3]). According to these investigations, competition in biofilms results in a stratified biofilm structure, the fast growing heterotrophic bacteria being placed in the outer layers, where both substrate concentration and detachment rate are high, while the slow growing nitrifying bacteria stay deeper inside the biofilm. Thus a heterotrophic layer can form above the nitrifiers in the biofilm, which constitutes a disadvantage to them when the bulk liquid oxygen concentration is low. In this case oxygen limitation resulting from consumption and resistance to mass transfer within the heterotrophic layer affects the nitrification performance negatively. As long as the bulk oxygen concentration is high enough to preclude its depletion in the biofilm, however, the heterotrophic layer can also have a positive effect on the nitrifiers by protecting them from detachment [4].

One possible approach to minimize the competition of heterotrophs and nitrifiers for oxygen is their spatial separation into a nitrifying biofilm population and a

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0043-1354/02/$ - see front matter
PII: S0043-1354(01)00229-9

heterotrophic population in suspension. This separation can be achieved by extending the reactor's hydraulic retention time. At comparatively long hydraulic retention times, the fast growing heterotrophic microorganisms (with reciprocal maximum specific growth rates smaller than the selected hydraulic retention time) grow mainly in suspension while the slow growing nitrifiers form biofilms [2]. However, in this study the reactor was operated at a high oxygen concentration of $6\,mg\,l^{-1}$, a condition which did also allow full nitrification in a conventional biofilm reactor (nitrifiers and heterotrophs in the biofilm) [2].

Reactors with a spatial separation of heterotrophic and nitrifying activity for the treatment of effluents with a high chemical oxygen demand (COD)/NH_4^+-N ratio can be very attractive for future practical applications if (i) it can be demonstrated that these reactors allow complete nitrification at a relatively low dissolved oxygen concentration and if (ii) the energy savings in aeration relatively to the traditional process compensate the investment costs in the construction of a bigger reactor required due to its longer retention time. So far the effects of influent composition and operational conditions were studied with respect either to total reactor performance (macroscale studies) or to biofilm composition and structure (microscale studies), but hardly to both simultaneously (for example [3]).

The research subjects of this study were the effects of different hydraulic retention times and changes in the organic carbon dosing on the population dynamics of nitrifying biofilm reactors operated at around $2\,mg\,l^{-1}$ dissolved oxygen. The specific objectives were: (i) to identify and quantify nitrifying and heterotrophic bacteria in the biofilm and in suspension using a set of rRNA-targeted oligonucleotide probes for fluorescence in situ hybridization (FISH), (ii) to correlate changes in microbial community composition in the biofilm and suspension with reactor performance during a shift from pure nitrification to combined nitrification and organic carbon removal, and (iii) to asses the recovery of the nitrification process after a shift back to pure nitrification.

2. Materials and methods

2.1. Biofilm reactors

Two laboratory-scale circulating bed reactors (CBR) of 1.2 l each [5] were employed for this study. This type of airlift reactor has a rectangular geometry of 310 mm height and $60\,mm \times 60\,mm$ cross-section and is separated in an up-flow aerated section and a down-flow non-aerated one by a vertical wall (Fig. 1). High density polyethylene granulate with a particle size of 1 mm and a density of $731\,kg\,m^{-3}$ was used as support material for biofilm growth. The superficial air velocity in both

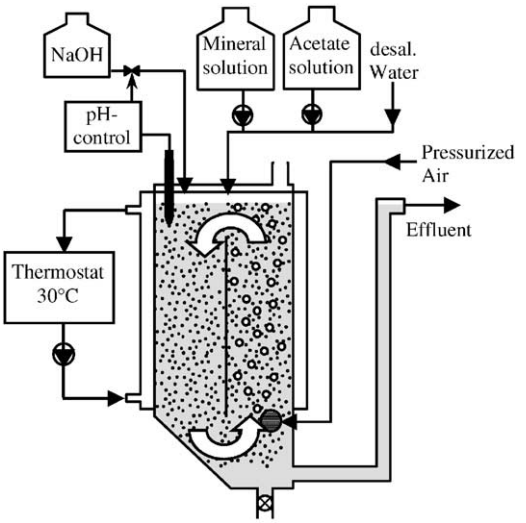

Fig. 1. Schematic diagram of continuous circulating bed reactor set-up.

reactors (defined as the air flow rate divided by the aerated reactor cross-section) was set at $0.003\,m\,s^{-1}$. The temperature was maintained at 30°C, and the pH was kept at 7.5 by adding sodium hydroxide (1 M). The experimental protocol included 4 phases (Fig. 2). The corresponding experimental conditions are summarized in Table 1.

Phase I. A CBR (hereafter reactor R_0) was filled with nitrifying biofilm particles (23 volume percent) obtained from a nitrifying circulating bed reactor, which was maintained at identical operating conditions. The biofilm particles had been stored at 4°C for 90 days prior to inoculation of reactor R_0. An ammonium solution was supplied continuously (N operation mode), with a retention time of 0.70 h. During phase I a stable nitrifying biofilm was established.

Phase II. Half of the biofilm particles from reactor R_0 were transferred to a second identical reactor. Both CBRs (hereafter reactors R_1 and R_2) were operated simultaneously with retention times of 0.8 h for reactor R_1 and 5.0 h for reactor R_2. The ammonium load supplied to each reactor was approximately half of the value used in phase I in order to maintain a constant NH_4^+-N load to biomass ratio ($0.46\,kg\,kg^{-1}\,d^{-1}$), and still no organic carbon was added (N operation mode).

Phase III. Acetate as an organic carbon source was supplied to reactors R_1 and R_2 at the same COD/NH_4^+-N mass ratio (C + N operation mode) in order to investigate the effect of organic carbon on the nitrification performance of reactors operating at different retention times.

Phase IV. Discontinuation of the supply of acetate to reactors R_1 and R_2 in order to study the recovery of the nitrification process (N operation mode).

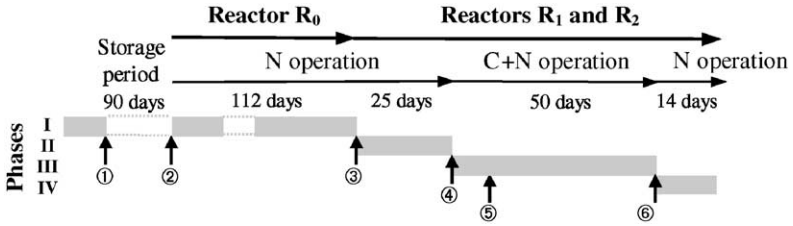

Fig. 2. Schematic diagram describing the experimental phases. The arrows indicate biofilm and suspended biomass sampling times.

Table 1
Operating conditions and performance of reactors R_0, R_1, and R_2. For all parameters average values are given for the different phases of operation

Phase/ mode of operation		Influent		NH_4^+-N load		NH_4^+-N removal (%)	COD load		COD removal (%)	COD/N applied ($g\,g^{-1}$)
		NH_4^+-N	COD ($kg\,m^{-3}$)	Applied	Removed ($kg\,m^{-3}\,d^{-1}$)		Applied	Removed ($kg\,m^{-3}\,d^{-1}$)		
Reactor R_0 hydraulic retention time of 0.7 h										
I	N	0.037	—[a]	1.26	1.20	95	—	—	—	—
Reactor R_1 hydraulic retention time of 0.8 h										
II	N	0.020	0.01	0.61	0.60	98	0.33	0.04	12	0.53
III	C+N	0.022	0.03	0.65	0.45	69	0.85	0.61	72	1.30
IV	N	0.019	0.01	0.57	0.51	90	0.17	0.05	29	0.30
Reactor R_2 hydraulic retention time of 5.0 h										
II	N	0.100	0.02	0.48	0.48	100	0.10	0.04	40	0.21
III	C+N	0.109	0.14	0.53	0.53→0	100→0	0.68	0.61	90	1.28
IV	N	0.117	0.02	0.56	0.53	94	0.08	0.05	63	0.14

[a] — = not determined.

Phases I, II and IV are characteristic of a pure nitrification process, while phase III corresponds to a combined nitrification and organic carbon removal process.

2.2. Media

During the experimental phases I, II and IV (N operation mode) $3.4\,ml\,min^{-1}$ of an ammonium medium was delivered to each reactor. The medium contained $(NH_4)_2SO_4$ ($0.59\,g\,l^{-1}$), $NaHCO_3$ ($0.19\,g\,l^{-1}$), KH_2PO_4 ($0.06\,g\,l^{-1}$), $CaCl_2\,2H_2O$ ($0.014\,g\,l^{-1}$) and trace elements. In reactors R_0 and R_1, also $15.9\,ml\,min^{-1}$ of deionized water was added to the reactors to obtain the desired hydraulic retention time. During phase III (C+N operation mode) $1.7\,ml\,min^{-1}$ of a sterilized acetate solution ($CH_3COONa\,3H_2O$, $0.71\,g\,l^{-1}$) was added separately to reactors R_1 and R_2. In order to maintain the retention time in both reactors constant, the ammonium medium's volumetric flow rate was reduced to half, and its concentration was doubled.

2.3. Overall kinetics

The macroscale reactor performance was evaluated from effluent samples filtered with 0.22 μm membrane filters. COD, ammonia plus ammonium, nitrite and nitrate were determined photometrically (LCK, Dr. Lange). Biofilm and suspended biomass total solids were measured according to APHA [6] using 0.22 μm membrane filters. Prior to this analysis, the biofilm was detached from the support material by an ultrasonic homogenizer (Bandelin electronics D-1000, Berlin) treatment (120 s at 50 W). The dissolved oxygen concentration was measured with an oxygen electrode (WTW, model Oxi 340-A).

2.4. Fluorescence in situ hybridization, microscopy and quantification of probe-targeted bacteria

Microbial population dynamics in biofilm particles and suspended biomass was evaluated using FISH with rRNA-targeted oligonucleotide probes. Samples 1–6

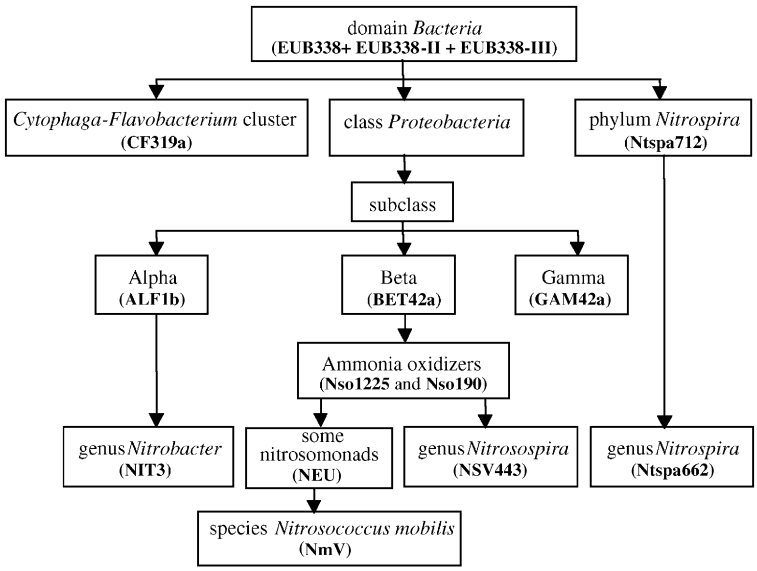

Fig. 3. Specificity of the rRNA-targeted oligonucleotide probes used for in situ identification of nitrifiers and heterotrophic bacteria.

(Fig. 2) were taken from the reactors and immediately fixed with paraformaldehyde. During phases I and II (N operation) only biofilm was sampled since the suspended biomass concentration was very low, while during phase III (acetate addition) both biofilm and suspended biomass samples were taken.

In situ characterization of microbial populations followed a top to bottom approach (Fig. 3). First the samples were hybridized with a probe set (EUB338, EUB338-II, EUB338-III) designed to target almost all bacteria [7], then with previously published group specific probes (Fig. 3; [8,9]). The ammonia-oxidizing and nitrite-oxidizing bacteria were identified using previously published probes (Fig. 3; [10–14]).

Oligonucleotide probes were purchased as derivatives labeled with the fluorescent dyes Cy3, Cy5, and 5(6)-carboxyfluorescein-N-hydroxysuccinimide-ester (FLUOS), respectively (Interactiva, Ulm, Germany). FISH was performed using the hybridization and washing buffers as described by Manz et al. [8]. A Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Jena, Germany) was used for image acquisition. For quantification of probe-targeted bacteria, simultaneous hybridizations were performed with Cy3 labeled specific probes and the FLUOS labeled bacterial probe set. The relative biovolume defined as the ratio between the area of probe-targeted bacteria to the area of all bacteria detectable by FISH was determined for each probe in 20 randomly recorded confocal images (thickness 1 μm) using the procedure described by Schmid et al. [15]. Biofilm thickness was determined for fresh, unfixed biofilm samples which were stained with a $0.25\,g\,l^{-1}$ fluorescein isothiocianate solution for 3 h at room temperature, using CLSM optical sectioning in the sagittal (*xz*) direction.

2.5. Comparative sequence analyses of the amoA gene

High resolution analyses of ammonia-oxidizer diversity in reactor R_0 was performed using the gene encoding the catalytic subunit of the ammonia-monooxygenase enzyme (*amoA*) as a marker. Amplification, cloning, sequencing and phylogenetic analyses of the biofilm-derived *amoA* fragments was performed as described by Purkhold et al. [16].

3. Results

3.1. Reactor performance

3.1.1. Phase I

One of the main drawbacks of biological nitrification processes is the requirement of a long start-up period. By using biofilm particles as inoculum, the start-up period of reactor R_0 in phase I could be reduced to 4 days after which the ammonium removal efficiency had reached 95%. Subsequently, R_0's biofilm particles were split between reactors R_1 and R_2 to ensure that both reactors had the same original microbial population composition.

3.1.2. Phases II to IV

Fig. 4 (A–D) depicts the performance of reactors R_1 and R_2 operated with retention times of 0.8 and 5.0 h, respectively, during pure nitrification (phases II and IV) and combined nitrification and organic carbon removal (phase III). Tables 1 and 2 summarize the experimental results obtained during reactors operation and the characterization of biofilm and suspended biomass. Though acetate as organic carbon source was only added during phase III, there was a certain background COD in the influent during phases I, II and IV (Table 1; Figs. 4B and D) deriving from oxidizable matter in the deionized water source. However, during these phases maximal 63% of the incoming COD was removed demonstrating that a significant fraction of these compounds were not degraded in the reactors.

During phase II, both reactors had a stable performance, no nitrite accumulation was observed and the NH_4^+-N effluent concentration was below $1.0\,mg\,l^{-1}$ corresponding to an ammonium removal efficiency higher than 95%. The biofilm characterization at the end of phase II showed similar biofilm thickness (41 and 42 μm), and biofilm mass concentration (2.48 and $2.43\,kg\,m^{-3}$) in reactors R_1 and R_2.

Shortly after the addition of acetate to reactor R_1 (start of phase III), the ammonium removal rate decreased from 0.65 to $0.45\,kg\,m^{-3}\,d^{-1}$ (69%) and hereafter, was constant (Fig. 4A). Due to a mechanical problem in the ammonium dosing pump, reactor R_1 received an ammonium overload 10 days after the start of phase III (gray area in Fig. 4A). The ammonium removal rate, however, remained constant. In reactor R_2

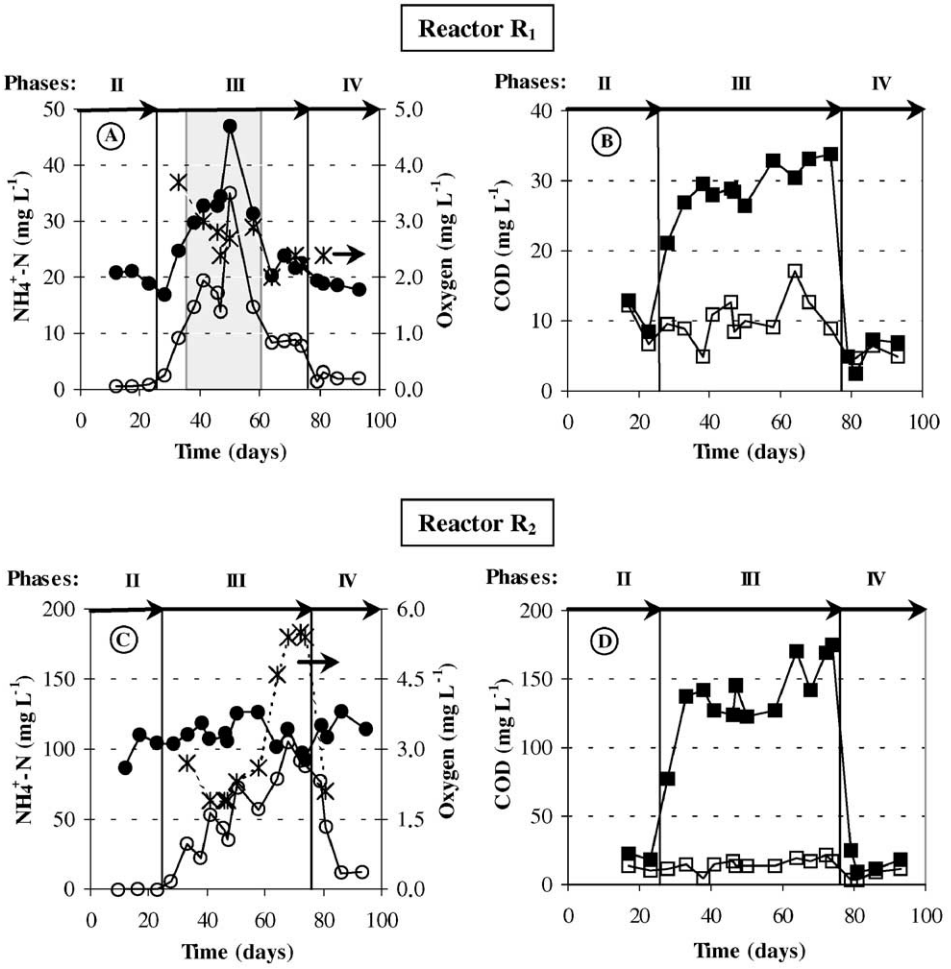

Fig. 4. Time changes of NH_4^+-N, COD and oxygen during operation of reactors R_1 and R_2 with pure nitrification (phases II and IV) and combined nitrification and organic carbon removal (phase III). Closed symbols correspond to influent concentrations and open symbols to effluent concentrations. *Corresponds to oxygen concentrations.

Table 2
Characterization of biofilm and suspended biomass in reactors R_0, R_1, and R_2 during the different phases of operation. Values listed in the table are the average ±95% confidence interval

Mode of operation/ sample		Biofilm mass per reactor volume ($kg\,m^{-3}$)	Suspended solids concentration ($kg\,m^{-3}$)	Biofilm thickness (μm)
Reactor R_0 hydraulic retention time of 0.7 h				
N	3	2.70±0.80	—[a]	33±5
Reactor R_1 hydraulic retention time of 0.8 h				
N	4	2.48±0.37	—	41±2
C+N	5	2.49±0.37	—	—
C+N	6	2.54±0.41	0.46±0.01	44±3
Reactor R_2 Hydraulic retention time of 5.0 h				
N	4	2.43±0.40	—	42±3
C+N	5	2.72±0.40	—	—
C+N	6	3.63±0.38	0.61±0.04	56±5

[a] — = not determined.

the ammonium removal continuously decreased to zero within 50 days (Fig. 4C), while the dissolved oxygen concentration simultaneously increased (Fig. 4B). A constant COD removal rate of $0.61\,kg\,m^{-3}\,d^{-1}$ was reached within 5 days in both reactors (Figs. 4B and D) and no nitrite accumulation was observed during the entire phase III. The increase of biofilm thickness and mass after carbon addition was more pronounced in reactor R_2 than in R_1 (Table 2).

The amount of biomass found in the reactors during phase III was by a factor of 10 to 40 higher than the value expected for theoretical equilibrium between bacteria growth rate and dilution rate (Table 2). This inconsistency was obviously caused by an accumulation of mostly heterotrophic biomass in the reactors' bottom and effluent tubes increasing the actual sludge retention time considerably. In reactor R_2 this effect was supported by the lower hydraulic retention time, leading to a higher biomass accumulation than in R_1. The accumulated biomass was removed once per week and included in the samples for suspended solids quantifications and FISH analyses of suspended cells.

After the discontinuation of acetate addition (phase IV) the ammonium removal in both reactors recovered and after a period of 14 days approx. 90% of the influent ammonium load was nitrified. In reactor R_2 the dissolved oxygen concentration decreased back to the value at the beginning of phase III.

3.2. Diversity of nitrifying bacteria in the reactors

The ammonia-oxidizing cells in all biofilm samples could be labeled simultaneously with probes BET42a, Nso1225 and Nso190. No ammonia-oxidizers belonging to the *Nitrosospira*-cluster were detected. A fraction of the ammonia-oxidizing population was detectable with probe NEU, while *Nitrosococcus mobilis* was absent. The NEU-positive subpopulation of ammonia-oxidizers is most likely affiliated with the *Nitrosomonas europaea/eutropha* group [17]. Comparative sequence analyses of *amoA* clones derived from the biofilm of reactor R_0 independently confirmed the presence of two different groups of ammonia-oxidizers (Fig. 5). One *amoA* sequence cluster is closely related to *Nitrosomonas europaea*, most likely representing the NEU-positive ammonia-oxidizers, while the other *amoA* cluster is not closely related with any described ammonia-oxidizer reference strain. In both reactors the nitrite-oxidizing cells in the biofilm were affiliated with the genus *Nitrospira* during all phases of operation. Members of the genus *Nitrobacter* were only detected in the biofilm from reactor R_2 (5.0 h retention time) during operation with acetate addition (Table 3).

3.3. Diversity of heterotrophic bacteria in the reactors

The heterotrophic bacteria present in the biofilm of reactor R_2 were *Proteobacteria* of the alpha- and beta-subclasses while in reactor R_1 only beta-subclass *Proteobacteria* could be detected (Table 3). In both reactors the microbial populations in the suspended biomass during phase III were *Proteobacteria* of the alpha-, beta- and gamma-subclasses and bacteria belonging to *Cytophaga-Flavobacterium*-cluster (Table 4).

The dominating microbial populations both in biofilm and suspended biomass during combined organic carbon and ammonia oxidation belong to the beta-subclass of *Proteobacteria*. Concerning the two reactor

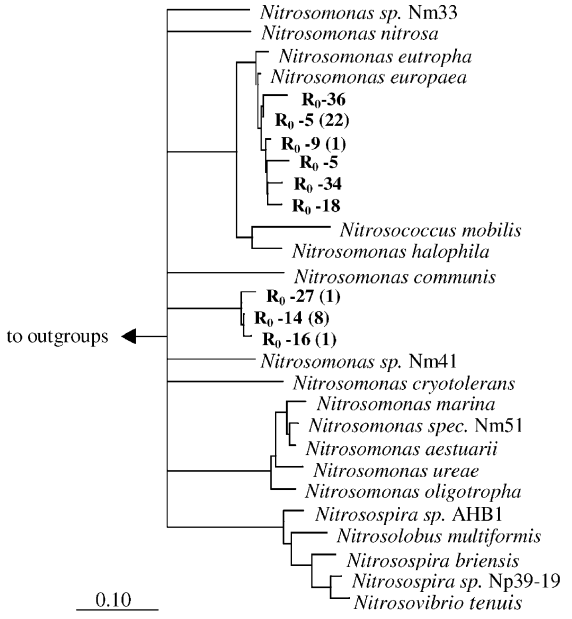

Fig. 5. Phylogenetic FITCH tree reflecting the relationships of the ammonia-oxidizers in reactor R_0 based on *amoA* sequences. The scale bar indicates the number of expected amino acid substitutions per site per unit of branch length. Numbers in brackets indicate the number of clones with almost identical sequences (>99% amino acid sequence similarity) which were retrieved from the reactor.

operation modes (N and C+N operation) the major difference is that during pure nitrification all beta-subclass *Proteobacteria* were ammonia-oxidizers, while during acetate dosing an additional presumably heterotrophic beta-subclass population developed. With respect to the different hydraulic retention times the major difference is a presumably heterotrophic alpha-subclass *Proteobacteria* population that occurred during pure nitrification only in reactor R_2 biofilm and disappeared after introduction of acetate.

3.4. Population dynamics in biofilm and suspended biomass

Values for each probe's targeted-bacteria are depicted in Table 3 for biofilm and in Table 4 for suspended biomass. The relative biovolumes of the different microbial populations (determined by FISH) were normalized by taking into account the differences in biomass (measured as dry weight) in the different reactors and samples. The FISH biovolume percentages for the sample with the highest biomass per reactor volume (sample 6) were kept unchanged and the FISH percentages of the other samples were reduced according to the biomass differences.

The fraction of the heterotrophic beta-*Proteobacteria* population (HET) present in the biofilm of reactors R_1 and R_2 can be determined as follows:

$$(\mathrm{Area}_{\mathrm{BET42a}})_{\mathrm{HET}} = \mathrm{Area}_{\mathrm{BET42a}} - \mathrm{Area}_{\mathrm{Nso1225}}. \quad (1)$$

Simultaneous hybridization of biofilm samples with probes ALF1b and Ntspa662 designed for specific detection of the alpha-subclass of *Proteobacteria* and the genus *Nitrospira*, respectively, demonstrated that members of the genus *Nitrospira* are non-specifically targeted by probe ALF1b. Consistent with this finding, a recent data base inspection demonstrated that *Nitrospira*-like 16S rRNA-sequences retrieved from wastewater treatment plants possess the full match target site of probe ALF1b. Consequently, *Nitrospiras* have to be included in the list of non-alpha-subclass *Proteobacteria* targeted by probe ALF1b [8]. The relative biovolume labeled with probe ALF1b was higher than the one labeled with probe Ntspa662 in biofilm samples taken from reactor R_2, except for the last biofilm sample during phase III (Table 3). This demonstrates that an ALF1b-positive population not related to *Nitrospira* appeared in the biofilm when the hydraulic retention time changed from 0.8 to 5.0 h, corresponding to the transition from reactor R_0 to reactor R_2 (phase II—N operation mode), and disappeared after the operation of reactor R_2 with acetate for 50 days (phase III—C+N operation mode).

The fraction of the alpha-*Proteobacteria* population (HET) developed in the biofilm from reactor R_2 during pure nitrification (phase II) can be estimated as follows:

$$(\mathrm{Area}_{\mathrm{ALF1b}})_{\mathrm{HET}} = \mathrm{Area}_{\mathrm{ALF1b}} - \mathrm{Area}_{\mathrm{Ntspa662}}. \quad (2)$$

The fraction of biofilm and suspended biomass bacteria identified with specific gene probes (F) in relation to all bacteria (EUB338 probe set) was calculated as listed below:

Biofilm samples

$$\mathrm{Area}_{\mathrm{ALF1b}} \approx \mathrm{Area}_{\mathrm{Ntspa662}} \Rightarrow \mathrm{F} = \mathrm{Area}_{\mathrm{BET42a}} + \mathrm{Area}_{\mathrm{Ntspa662}}, \quad (3)$$

$$\mathrm{Area}_{\mathrm{ALF1b}} > \mathrm{Area}_{\mathrm{Ntspa662}} \Rightarrow \mathrm{F} = \mathrm{Area}_{\mathrm{BET42a}} + \mathrm{Area}_{\mathrm{ALF1b}}. \quad (4)$$

Suspended biomass samples

$$\mathrm{F} = \mathrm{Area}_{\mathrm{ALF1b}} + \mathrm{Area}_{\mathrm{BET42a}} + \mathrm{Area}_{\mathrm{GAM42a}} + \mathrm{Area}_{\mathrm{CF319a}}. \quad (5)$$

The population dynamics of nitrifiers and heterotrophs in the biofilm and suspended biomass from reactors R_0, R_1 and R_2 during the different phases of operation is depicted in Fig. 6. For most biofilm and suspension samples, more than 80% of the bacteria detectable by the EUB338 probe set could simulta-

Table 3
Microbial community composition of reactors R_0, R_1 and R_2 biofilm during the different phases of operation[a]

Sample/reactor	NF	Oligonucleotide probes						F
		ALF1b	BET42a	Nso1225	NEU	Ntspa662	NIT3	
Phase I—N removal								
1 R_0	0.74		**39**	**39**	**25**	**38**	**0**	105
		—	(53±4)	(53±4)	(34±3)	(52±4)	(0)	
2 R_0		—	—	—	(56±5)	(30±5)	0	—
3 R_0	0.74	**20**	**41**	**41**	**19**	**20**	**0**	82
		(27±3)	(55±4)	(55±4)	(26±3)	(27±3)	(0)	
Phase II—N removal								
4 R_1	0.68	**21**	**30**	**30**	**16**	**23**	**0**	78
		(31±3)	(45±6)	(45±6)	(24±6)	(33±5)	(0)	
R_2	0.67	**35**	**21**	**21**	**14**	**27**	**0**	83
		(52±3)	(31±5)	(31±5)	(21±3)	(41±2)	(0)	
Phase III—C + N removal								
5 R_1	0.68	**12**	**26**	**19**	**12**	**14**	**0**	59
		(18±3)	(39±5)	(27±3)	(17±3)	(21±2)	(0)	
R_2	0.75	**30**	**29**	**21**	**19**	**23**	**0**	80
		(40±7)	(39±5)	(28±7)	(25±5)	(30±6)	(0)	
6 R_1	0.70	**24**	**34**	**21**	**5**	**22**	**0**	81
		(34±6)	(49±8)	(30±4)	(7±2)	(32±5)	(0)	
R_2	1.00	**29**	**60**	**0**	**0**	**31**	**3**	94
		(29±6)	(60±4)	(0)	(0)	(31±6)	(3±1)	

[a] The relative biovolumes of probe-defined bacterial populations (values in brackets) and the respective normalized values in regard to the biomass content of the samples (bold values) are given in columns 3–8. No normalized values are given for sample 2 since the biomass content was not determined for this sample. The fraction of bacteria detectable with the bacterial probe set, which were identified with specific oligonucleotide probes, is given in column 9. Values listed in the table are the average percentage ±95% confidence interval. For all biofilm samples no signals were observed with probes specific for the *Cytophaga-Flavobacterium*-cluster (CF319a) and the gamma-subclass of *Proteobacteria* (GAM42a), respectively. NF = normalization factor; — = not determined.

Table 4
Microbial community composition of reactors R_1 and R_2 suspended biomass during combined nitrification and organic carbon removal (columns 3–6)[a]

Sample/reactor	NF	Oligonucleotide probes				F
		ALF1b	BET42a	GAM42a	CF319a	
Phase III—C + N removal						
5 R_1	0.18	**2.2**	**11.5**	**0**	**0.2**	76
		(12±1)	(64±2)	(0)	(1±0)	
R_2	0.17	**1.6**	**14**	**0.5**	**0.9**	99
		(10±1)	(81±1)	(3±0)	(5±1)	
6 R_1	0.18	**3.1**	**14.6**	**0.2**	**0.2**	100
		(17±3)	(81±2)	(1±0)	(1±0)	
R_2	0.17	**1.2**	**13**	**1.9**	**1.5**	104
		(7±1)	(77±4)	(11±1)	(9±2)	

[a] Column 7 displays the fraction of bacteria detectable with the bacterial probe set which were identified with specific oligonucleotide probes. Values listed in the table are the average percentage ±95% confidence interval (values in brackets). NF = normalization factor.

neously be classified by at least one of the more specific probes applied (Tables 3 and 4; Eqs. 3–5). However, this does not hold true for the biofilm and suspended biomass samples taken from reactor R_1 during the accidental overloading period with ammonium (sample 5). In this sample 41% of the biofilm bacteria and 24%

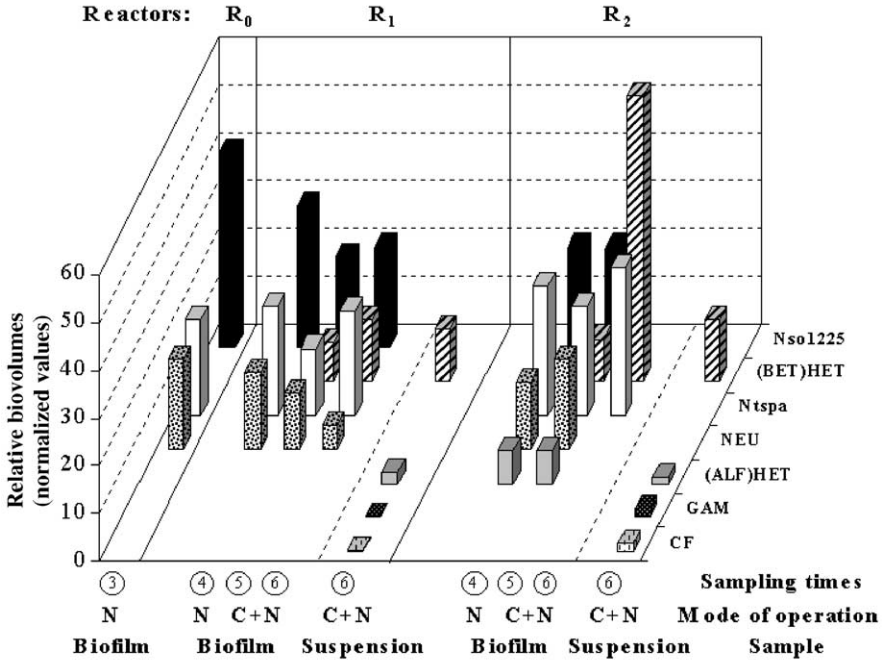

Fig. 6. Population dynamics of ammonia-oxidizers, nitrite-oxidizers and heterotrophs in the biofilm during process operation of reactors R_1 and R_2 with pure nitrification [phase I (sampling point 3) and phase II (sampling point 4)] and combined nitrification and organic carbon removal (phase III—sampling points 5 and 6). In addition, the microbial community composition of the suspended biomass is displayed for both reactors for sampling point 6.

of the suspended bacteria which were detectable by FISH could not further be classified with the more specific probes. Whether these bacteria appeared in the reactor due to the acetate addition or the elevated ammonia concentrations could not be clarified.

3.5. Population dynamics during phase I

Biofilm samples were taken before and after the storage period preceding operation of reactor R_0 (samples 1 and 2) and after 112 days of operation (sample 3), in order to evaluate the effects of the storage period on the microbial community composition.

The samples were hybridized in situ with probes Nso1225, NEU and Ntspa662, as well as the EUB338 probe set. After the storage period (sample 2) the fraction of ammonia-oxidizers was larger than during normal operation (samples 1 and 3) while the relative abundance of nitrite-oxidizers decreased (data not shown), suggesting that the nitrite-oxidizers decayed faster than the ammonia-oxidizers under the selected storage conditions. The characterization of the biofilm from reactor R_0 during operation (sample 3) showed that the ammonia-oxidizing population detected with probe Nso1225 was able to recover a comparable relative abundance as before the storage period (sample 1), while the nitrite-oxidizing population decreased considerably from 38% (sample 1) to 20% (sample 3). Furthermore, the morphology of the nitrite-oxidizing clusters changed from big clusters present before the storage period (sample 1), to small ones mixed with net-like structures afterwards (sample 3). Despite the decrease in the relative abundance of nitrite-oxidizers, no nitrite accumulation was observed during phase I. This suggests that the nitrite-oxidizers present in net-like structures in sample 3 are more active (possibly due to a better accessibility to substrates) than those occurring in big clusters in sample 1.

3.6. Population dynamics during phase II

In the transition from phases I to II the amount of biofilm particles from reactor R_0 was split up between reactors R_1 and R_2, while the NH_4^+-N load per reactor was reduced from 1.24 to 0.48–0.61 $kg\,m^{-3}\,d^{-1}$. Despite the lower amount of biofilm particles in R_1 and R_2 than in R_0, the biomass concentration per reactor volume was similar in both reactors during phases I and II (Table 2). This can be explained by a lower biofilm detachment rate in reactors R_1 and R_2 caused by a decrease in the

collision frequency between biofilm particles. Consequently, the NH_4^+-N load to biomass ratio was higher in R_0 ($0.46\,kg\,kg^{-1}\,d^{-1}$) than in R_1 ($0.25\,kg\,kg^{-1}\,d^{-1}$) and R_2 ($0.24\,kg\,kg^{-1}\,d^{-1}$). As a result the effluent NH_4^+-N concentration of the reactors dropped from $1.8\,mg\,l^{-1}$ (R_0) to $0.6\,mg\,l^{-1}$ (R_1) and $0.2\,mg\,l^{-1}$ (R_2), respectively. The normalized abundance of ammonia-oxidizers in the biofilm decreased from reactor R_0 (sample 3) to reactor R_1 (sample 4) and reactor R_2 (sample 4), respectively (Fig. 6; Table 3). The NH_4^+-N concentration in reactors R_1 and R_2 was lower than the assumed saturation constant of $0.7\,mg\,l^{-1}$ [18], and thus most likely limited the amount of ammonia-oxidizers in the biofilm. Unlike the ammonia-oxidizing population the relative abundance of nitrite-oxidizers increased in R_2 and remained approximately constant in R_1 during phase II (N operation). According to Eq. (2), in reactor R_2 a heterotrophic population (8%) detectable with probe ALF1b developed.

3.7. Population dynamics during phase III

The newly introduced supply of acetate during phase III induced the growth of heterotrophic microorganisms both in suspension and in the biofilm in both reactors. Biofilm samples collected 12 days after acetate addition (sample 5) showed the presence of a thin layer of heterotrophic beta-*Proteobacteria* distributed discontinuously on top of the nitrifying biofilm, accounting for 7–8% of the bacterial biofilm population in both reactors. Like during phase II, a heterotrophic population detectable with probe ALF1b (7%) was exclusively present in reactor R_2, 12 days after addition of acetate. During the first 12 days of phase III the abundance of nitrite-oxidizers decreased in the biofilm of both reactors, while the ammonia-oxidizers decreased only in R_1. The latter observation corresponds with the higher loss of ammonium removal observed in reactor R_1, (28% loss) compared to reactor R_2 (19% loss).

The ammonium removal in reactor R_2 continued to decrease during the subsequent days of acetate addition until a complete breakdown of the nitrification process happened. After 50 days of acetate addition (sample 6), no ammonia-oxidizing bacteria could be detected in reactor R_2 while the relative in situ abundance of *Nitrospira*-like nitrite-oxidizers was similar to the one during phase II (N operation). At that time reactor R_2 biofilm was dominated by heterotrophic beta-*Proteobacteria* that amounted to 60% of the total biovolume labeled with the bacterial probe set. In contrast to reactor R_2, the extended addition of acetate did not cause a complete breakdown of nitrification in reactor R_1. In this reactor, the ammonium removal efficiency stabilized at 69% and the heterotrophic beta population in the biofilm amounted to a maximum biovolume fraction of only 13% (sample 6). As in reactor R_2, the nitrite-oxidizing population in reactor R_1 did recover its initial relative abundance during prolonged acetate dosage.

4. Discussion

In this study the microbial community composition and dynamics in two nitrifying biofilm reactors (differing in hydraulic retention time) was monitored using molecular tools during a shift in process operation from pure nitrification to combined nitrification and organic carbon removal. In general, the dynamics of the microbial communities correlated well with the performance of the respective reactors. In the following sections several interesting findings are discussed in more detail.

4.1. Composition and dynamics of bacterial populations in the reactors

In both reactors at least two populations of beta-subclass ammonia-oxidizers were present. As demonstrated by oligonucleotide probing and comparative AmoA sequence analysis, one of these populations was closely related to the model organism *Nitrosomonas europaea*, while the other population surprisingly showed no close relationship with recognized ammonia-oxidizers. Nitrite oxidation was catalyzed in both reactors mainly by *Nitrospira*-like bacteria confirming the recently recognized importance of these bacteria for nitrite oxidation in several environments (e.g. [13,14,19]). In both reactors Nitrospiras occurred in previously not observed net-like structures in the biofilm. In the present work, *Nitrobacter* could only be detected in small numbers in the biofilm of reactor R_2 during simultaneous addition of acetate and ammonium (phase III) and a concurrent increase of the NO_2^--N concentration from $0.02\,mg\,l^{-1}$ (phase II) to $0.39\,mg\,l^{-1}$ (phase III). This result is consistent with the recently published hypothesis that Nitrospiras are k-strategists (and thus thrive at low nitrite concentrations) while *Nitrobacter* as r-strategist can compete successfully only in environments with relatively high nitrite concentrations [19]. The absence of detectable Nitrobacters in reactor R_1 during phase III might have been caused by the lower NO_2^--N accumulation ($0.22\,mg\,l^{-1}$) in this reactor compared to reactor R_2. In addition, *Nitrobacter* might have benefited in phase III in R_2 from its capability to grow mixotrophically with acetate while Nitrospiras might not possess this capability [14]. On the other hand we yet have no explanation for the observed increase of *Nitrospira*-like nitrite oxidizers during phase III in both reactors despite the decreasing or even failing nitrification during this period.

Despite the lack of organic carbon dosing during phase II a presumably heterotrophic population affiliated with the alpha-subclass *Proteobacteria* was present in the biofilm of reactor R_2 (5.0 h retention time), but disappeared during reactor operation with acetate (end of phase III). Most likely this population was growing on soluble microbial products (SMP) formed by active nitrifiers in the biofilm [20] since SMP concentration should be higher in reactor R_2 than in reactor R_1 due to the difference in retention time. In phase III the supply of acetate provoked the breakdown of the nitrification process in reactor R_2, consequently SMP were no longer produced and the alpha-proteobacterial population disappeared from the biofilm.

During combined nitrification and organic carbon removal in phase III a higher group-level diversity of heterotrophic microorganisms was found in suspension than in the biofilm. This result might be explained by the fact that the heterotrophic biofilm community is exposed to a higher shear stress compared to the suspended consortia. Thus, only fast growing heterotrophs can maintain themselves in the outer layer of the biofilm, a selection pressure which might reduce diversity.

4.2. Population dynamics versus reactor performance

During phase II (pure nitrification) the biofilm was, as expected, dominated by ammonia- and nitrite-oxidizing bacteria in both reactors. After acetate addition (phase III), the formation of a thicker layer of heterotrophic bacteria in reactor R_2 (with long retention time) on the surface of the nitrifying biofilm led to increased oxygen mass transfer resistance from bulk liquid to the nitrifiers. This coincided with a drastic reduction in the ammonia-oxidizing population and a subsequent breakdown of the nitrification process. In contrast, reactor R_1 operated with short retention time displayed a smaller increase of heterotrophic biofilm bacteria on the surface which corresponded to the less pronounced reduction in nitrification performance. An alternative hypotheses for the breakdown of the nitrification in reactor R_2 during phase III would be that nitrifiers but not the heterotrophs were lost from the biofilm due to detachment and finally washed out of the reactor. This would be consistent with the absence of in situ detectable ammonia-oxidizers in sample 6 of reactor R_2. However, there are two lines of evidence that oxygen limitation and not selective washout caused the nitrification breakdown. Firstly, the even slower growing nitrite-oxidizers still were detected in significant amounts (31%) within the biofilm of reactor R_2 at the end of phase III. Secondly, the re-establishment of 90% of the nitrification capacity within 14 days after stop of acetate addition (Fig. 4; phase IV) in reactor R_2 contradicts the possibility of a previous complete depletion of ammonia-oxidizers. The mass accumulation rate for nitrifying biofilm of $0.03\,kg\,m^{-3}\,d^{-1}$, [21] excludes the chance of such a fast recovery by re-growth. Keeping in mind previously published data [2] our findings were unexpected since we assumed that reactor R_2 will, due to its longer retention time, favor suspended growth of the fast reproducing heterotrophs and thus allow for a higher biofilm-mediated nitrification during the presence of acetate.

What remains to be discussed is (i) why a thicker heterotrophic biofilm is formed on top of the nitrifying biofilm in reactor R_2 compared to reactor R_1 and (ii) why heterotrophic biofilm formation and subsequent loss of nitrifying capacity was not observed in the airlift reactor of [2] after addition of organic carbon. Heterotrophic biofilm formation in reactor R_2 could be explained by increased liquid phase viscosity in this reactor due to the presence of extracellular biopolymers which accumulated compared to reactor R_1 due to the increased liquid retention time in reactor R_2. Increased liquid viscosity will lead to a stronger air bubble coalescence, thus decreasing the volume occupied by the gas phase, reducing the driving force for the circulation and ultimately the shear stress for the biofilm ([22,23]). Consistent with this argumentation, surface protuberances were observed by us microscopically in the biofilm of R_2 (reflecting the lower shear stress) while the biofilm of reactor R_1 was characterized by a much smoother biofilm surface (indicative for a higher shear stress [23]). Differences in liquid phase viscosity or other factors influencing turbulence and thus shear stress and substrate availability (e.g. reactor geometry, friction, structure of the biofilm support particles etc.) could also be responsible for the inconsistent results between this study and the work of van Benthum and co-workers. Independent from the actual reason(s) causing these inconsistent results of both studies, it is important to note that extension of hydraulic retention time caused dramatically different effects in similar biofilm reactors.

5. Conclusions

The following main conclusions can be drawn from the present study:

1. No major effect of the hydraulic retention time on the diversity of nitrifying bacteria in the biofilm was observed. A group of ammonia-oxidizers not closely related to any described reference strain was identified in the biofilm from both reactors showing that even in systems working with defined conditions, the bacterial diversity is not completely described.
2. Combined nitrification and carbon removal under oxygen limiting conditions could be accomplished in the biofilm reactor with low hydraulic retention time but failed in the reactor with high hydraulic retention

time. This unexpected finding was caused by the formation of a thick heterotrophic layer on top of the nitrifying biofilm in the latter reactor that limited the nitrifiers' oxygen supply. Thus, extension of the hydraulic retention time is not always sufficient to improve combined nitrification and organic carbon removal in biofilm reactors.

3. Today, the battery of molecular tools allows to precisely determine ecological key parameters of complex microbial communities present in engineered systems. In addition to species richness and evenness also the in situ activity of probe identified bacteria can be analyzed [24]. Future interdisciplinary research at the interface between molecular microbial ecology and civil engineering will almost certainly allow for a detailed understanding of the links between microbial diversity, process efficiency and process stability. For example, it should be possible by the use of molecular methods to define operational parameters which selectively increase the diversity within important functional groups of bacteria (e.g. nitrifiers) and thus render the microbial community more resistant against perturbations. Armed with such knowledge innovative strategies for process control and design as well as for bioaugmentation can be developed.

Acknowledgements

This work was supported by the Portuguese Ministry of Science and Technology (PRAXIS XXI BD/15943/98), the Deutscher Akademischer Austauschdienst (A/99/06961) and the European Comission (T.M.R. program, BioToBio project). Ulrike Purkhold was supported by Sonderforschungsbereich 411 from the Deutsche Forschungsgemeinschaft (Research Center for Fundamental Studies of Aerobic Biological Wastewater Treatment; project A2).

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