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COMBINED NITRIFICATION AND DENITRIFICATION IN A SINGLE AERATED REACTOR USING THE AEROBIC DENITRIFIER *COMAMONAS* SP. STRAIN SGLY2

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Abstract—A mixed culture of an aerobic denitrifier, named SGLY2, and autotrophic nitrifiers was carried out either in batch culture or in a single continuous, stirred aerobic reactor. The batch assays were used to show the effect of different carbon sources on the nitrifying activity, and to determine the optimum conditions to maintain the strain SGLY2 in the complex microflora. In particular, conditions of discontinuous addition of the carbon source, as well as nitrifying and denitrifying activities were observed under continuous culture with aerobic reduction of the N-oxides produced by autotrophs into nitrous oxide and dinitrogen. © 1997 Elsevier Science Ltd.

Key words—denitrification, anoxia, nitrification, aerobiosis

INTRODUCTION

Nitrification and denitrification are the two main steps of recycling ammonia to dinitrogen in the nitrogen cycle. Nitrification, defined as the oxidation of reduced inorganic nitrogen compounds, is realized by autotrophic aerobic microorganisms (Alexander *et al.*, 1960). Denitrification is a form of respiration in which oxidized nitrogen compounds are reduced to gaseous nitrogen compounds. It is realized by autotrophic or heterotrophic aerobic microorganisms able to use N-oxides as alternative electron acceptors to oxygen.

Conventional systems for the treatment of nitrogen-containing wastes are based on the principle that nitrification is an aerobic process, whereas denitrification is restricted to anoxic conditions. This implies spatial separation of nitrifying and denitrifying units, or temporal separation of each step by alternating aeration and no aeration in the same unit. However, for 10 years, some authors have shown unusual behaviour of nitrifiers and denitrifiers. Under oxygen-limited or anoxic conditions, autotrophic nitrifiers are able to reduce nitrate and/or nitrite to nitric or nitrous oxide and nitrogen gas, depending on the presence of a suitable electron donor (Poth, 1986; Remde and Conrad, 1990; Abeliovich and Vonshak, 1992; Bock *et al.*, 1995). A newly discovered process, called the Anammox process, consists of biological conversion of ammonium to dinitrogen under anoxic conditions, with nitrate or nitrite as the electron acceptors (Van de Graaf *et al.*, 1995). In a number of species, denitrification may proceed at a substantial rate under aerobic conditions, and in particular cases even at dissolved oxygen concentrations close to 7 $mg.l^{-1}$. In these bacteria, oxygen and nitrate are co-respired (Robertson and Kuenen, 1984, 1990; Lloyd *et al.*, 1987; Bonin and Gilewicz, 1991). The most studied aerobic denitrifier is *Thiosphaera pantotropha*, which exhibits an aerobic denitrification rate (at around 5.6 $mg.l^{-1}$ of dissolved oxygen) equivalent to 50% of that calculated under anoxic conditions (Robertson *et al.*, 1988). Microorganisms that are able to denitrify under high dissolved oxygen concentrations (e.g. *Thiosphaera pantotropha*, *Alcaligenes faecalis*) have also been found to be capable of heterotrophic nitrification (Robertson *et al.*, 1989). This implies the complete conversion of ammoniacal pollution to nitrogen gas by a single microorganism.

Existence of this diversity of behaviour allows us to propose new processes where nitrification and denitrification could take place in the same unit. A denitrifying bacterium, named SGLY2, isolated from an upflow anoxic filter has been reported to exhibit aerobic denitrifying potential in continuous culture at dissolved oxygen concentration close to 33 $mg.l^{-1}$ (Patureau *et al.*, 1995b). Aerobic denitrification in this strain is accounted for by a constitutive, periplasmic nitrate reductase (Patureau *et al.*, 1995a). In opposition to the other aerobic denitrifiers, the strain SGLY2 is unable to nitrify. In this paper, we describe the behaviour and the denitrifying performances of the strain SGLY2 in co-culture with autotrophic nitrifiers, stemming from a piggery

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wastewater treatment plant. These experiments were carried out in batch cultures using a synthetic mineral medium added to different carbon sources in order to allow the growth of the heterotroph. To measure directly dissolved oxygen concentration and gas production, a continuous co-culture was then realized using the same synthetic mineral feeding medium and succinate as carbon source for the strain SGLY2.

MATERIALS AND METHODS

Organism and medium

Isolation and characterization of the strain SGLY2, used in this study, have been described in detail elsewhere (Patureau *et al.*, 1994). The autotrophic microflora was grown on a synthetic mineral medium made up of the following for 1 l: 3 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.05 g $MgSO_4$, 4 mg $CaCl_2$, 25 ml cresol red solution 0.0005% and 0.1 ml of iron solution (containing for 1 l: 1.4 g disodium EDTA, 5 g $FeSO_4.7(H_2O)$ and 0.5 ml concentrated sulphuric acid). Calcium and magnesium salts were separately autoclaved to avoid precipitation. During growth, pH was maintained at 8.2 by addition of sterile 50% potassium carbonate. Cells of SGLY2 were continuously grown on a synthetic medium (described in Patureau *et al.*, 1995b) with succinate as carbon source under aerobic conditions. A 200 ml portion of the cellular suspension was harvested by centrifugation, washed twice with sterile phosphate buffer (0.01 M, pH 7) and concentrated in the same solution. This concentrated cell suspension was then used to inoculate the different nitrifying cultures.

Culture conditions

Batch cultures were carried out in 500 ml Erlenmeyer flask filled with 100 ml of the synthetic mineral medium. These flasks were shaken in a rotary shaker (250 rpm) at 30°C to maintain an homogenous culture and to allow better gas exchange. Six Erlenmeyer flasks were inoculated with 100 ml of the autotrophic microflora:

- one control flask;
- another one used to test the effect of addition of the strain SGLY2;
- four other flasks used to test, on one hand, the effect of addition of different carbon sources (succinate, ethanol, caproate and yeast extract), and on the other hand the combined effect of the carbon source and of the strain.

The carbon source and the cellular suspension of SGLY2 were added to reach a final concentration of 100 $mg.l^{-1}$ of C-carbon and 10 $mg.l^{-1}$ of protein, respectively. After the complete consumption of ammonium in the flasks, a known amount of sterile solution of ammonium sulphate was again added to reach 300–600 $mg.l^{-1}$ of $N\text{-}NH_4^+$. At the beginning of each cycle, the pure strain was added with the corresponding carbon source. The latter was added daily to maintain the denitrifying activity. For each supply of $N\text{-}NH_4^+$, a global nitrogen balance was calculated by considering the disappearance of $N\text{-}NH_4^+$. As we are in batch assay, this consumption of ammonium is equivalent to $N\text{-}NO_x$ production, plus nitrogen assimilation, plus nitrogen stripping.

The continuous culture was realized in a 2 l Biolafitte reactor (Patureau *et al.*, 1995b), filled with 1.5 ml of the mineral medium. The medium was stirred at a constant rate of 700 rpm and temperature was regulated at 30°C. pH was measured using an Ingold pH electrode, connected to an Ingold transmitter 2300 and was maintained at 8 by the addition of sterile 50% K_2CO_3. The dissolved oxygen concentrations were measured by a polarographic type electrode. Aerobic conditions were obtained by sparging the medium with pure oxygen (quality C oxygen, Alphagas). The dissolved oxygen concentration was maintained at around 14 $mg.l^{-1}$, implying non-limited conditions for autotrophs and heterotrophs growth. The feeding medium was sparged with argon to avoid nitrogen entrance, which could cause errors in the calculation of the nitrogen balance. The experiments started by inoculating the 100 ml of mineral medium with 100 ml of the autotrophic microflora, by allowing the culture to grow and nitrify, and by feeding the reactor to reach the working volume of 1.5 l. After stabilization of the continuous nitrifying culture, some parameters were tested: different types of addition of carbon source with or without addition of the pure strain, and different concentrations of carbon and of the cellular suspension of the pure strain.

The different microflora were followed by spreading an appropriate dilution of the cellular suspension on different selective media: the mineral agar medium for the autotrophs, a nitrate–succinate agar medium under anoxic conditions for the strain SGLY2 and a nutrient agar medium for the heterotrophs.

Under continuous culture, nitrogen balances and different percentages were performed on the mass of the components resulting from considering both the flow and the reactor volume, because the system never reaches a steady-state (balance between day $j-1$ and day j), considering that in the reactor, evolution of the different components is linear and, therefore, between $j-1$ and j, averages

$$[N]_e = \frac{[N]_{e,j-1} + [N]_{e,j}}{2};$$

$$A = [\text{N-NH}_4]_{i,j-1} \cdot \frac{Q}{V}$$

$$B = \frac{([\text{N-NH}_4]_{e,j} + [\text{N-NO}_x]_{e,j} + [\text{N-Protein}]_{e,j} + [\text{N-NH}_4]_{e,j-1} + [\text{N-NO}_x]_{e,j-1} + [\text{N-Protein}]_{e,j-1})}{2} \cdot \frac{Q}{V}$$

$$C = \frac{([\text{N-NH}_4]_{e,j} + [\text{N-NO}_x]_{e,j} + [\text{N-Protein}]_{e,j} - [\text{N-NH}_4]_{e,j-1} - [\text{N-NO}_x]_{e,j-1} - [\text{N-Protein}]_{e,j-1})}{\Delta j}$$

Balance = $A - B - C$ (i = influent, e = effluent, Q = feeding delivery, V = working volume).

According to the results obtained with the discontinuous cultures, the quantity of nitrogen stripped out of the system as NH_3 can be considered to be negligible.

- The percentage of apparent nitrification = AN (1)

$$= \frac{\left\{\frac{([\text{N-NO}_x]_{e,j} + [\text{N-NO}_x]_{e,j-1})}{2} \cdot \frac{Q}{V}\right\} + \left\{\frac{([\text{N-NO}_x]_{e,j} - [\text{N-NO}_x]_{e,j-1})}{\Delta j}\right\}}{[\text{N-NH}_4]_{i,j-1} \cdot \frac{Q}{V}}$$

• The percentage of assimilation = AS (2)

$$= \frac{\left\{\frac{([\text{N-Protein}]_{e,j} + [\text{N-Protein}]_{e,j-1})}{2} \cdot \frac{Q}{V}\right\} + \left\{\frac{([\text{N-Protein}]_{e,j} - [\text{N-Protein}]_{e,j-1})}{\Delta j}\right\}}{[\text{N-NH}_4]_{i,j-1} \cdot \frac{Q}{V}}$$

• The percentage of residual ammonium = AR (3)

$$= \frac{\left\{\frac{([\text{N-NH}_4]_{e,j} + [\text{N-NH}_4]_{e,j-1})}{2} \cdot \frac{Q}{V}\right\} + \left\{\frac{([\text{N-NH}_4]_{e,j} - [\text{N-NH}_4]_{e,j-1})}{\Delta j}\right\}}{[\text{N-NH}_4]_{i,j-1} \cdot \frac{Q}{V}}$$

• The percentage of effective nitrification = EN (4)

$$= \frac{([\text{N-NH}_4]_{e,j} + [\text{N-Protein}]_{e,j} + [\text{N-NH}_4]_{e,j-1} + [\text{N-Protein}]_{e,j-1})}{2} \cdot \frac{Q}{V} = Y_1$$

$$= \frac{([\text{N-NH}_4]_{e,j} + [\text{N-Protein}]_{e,j} - [\text{N-NH}_4]_{e,j-1} - [\text{N-Protein}]_{e,j-1})}{\Delta j} = Y_2$$

$$(4) = \left([\text{N-NH}_4]_{i,j-1} \cdot \frac{Q}{V}\right) - \left(\frac{Y_1 + Y_2}{[\text{N-NH}_4]_{i,j-1} \cdot \frac{Q}{V}}\right)$$

• The percentage of denitrification = D = ((4) − (1)) · 100/(4); NE and D were calculated only in the case of a positive balance.

Analytical measurements

The liquid samples were centrifuged at 17,500 *g* and 4°C for 15 min. The supernatants were diluted as required for the different analyses. Ammonium was determined using a Büchi 320 apparatus, according to the method recommended by Rodier (1975). Nitrate and nitrite were measured by an exchange ion chromatography system using conductivity detection (DIONEX-100). Separation and elution of the anions were carried out on a IonPacAS4A Analytical Column using a carbonate–bicarbonate eluent and a sulphuric acid regenerant.

Protein concentrations were determined by Lowry's procedure (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Succinate concentrations were determined by high performance liquid chromatography on a Biorad Aminex HPX-87-H column, maintained at 35°C, using a refractometric detector (45°C). Elution was carried out with sulphuric acid (N/100).

Ethanol was measured by gas chromatography, with nitrogen as carrier gas, using a flame ionization detector. Gas composition was analysed by gas chromatography with a Shimadzu GC-8A apparatus with argon as carrier gas, using a katharometer detector. Carbon dioxide and nitrous oxide were separated on a Hayesep Q column (80–100 mesh, 2.0 m × 1/8 in). Oxygen and nitrogen were separated on a molecular sieve column 5A (80–100 mesh, 2.0 m × 1/8 in). The injector and detector temperature was 100°C. The column temperature was 35°C.

RESULTS

Influence of the carbon source on nitrifying activity in batch cultures

During week 1, the six Erlenmeyer flasks were maintained under nitrifying conditions to allow stabilization of the microflora. During this week, in each flask, there was consumption of ammonium without the production of N-NO_x and biomass. In these cases, there was surely nitrogen stripping out due to an increase of the pH link to biomass turnover. This lag phase corresponded to the development of the nitrifying microflora. After this period, in the control flask, the consumption of ammonium was equivalent to N-NO_x production and nitrogen assimilation at 1 or 2%. It seemed that under these conditions of growth and activity of the nitrifying biomass, there was no more nitrogen stripping. That is why this term was not considered in the nitrogen balance calculation. In the other flasks, before the addition of the strain SGLY2 and/or carbon, the overall amount of ammonium consumed was transformed to N-NO_x and biomass (Table 1 with succinate period 1).

The first experiment studied the effect of succinate, ethanol, caproate and yeast extract addition on the nitrifying activity, compared to the behaviour of the control flask. Addition of carbon enhanced an increase in heterotrophic biomass concentration from 10^4 cells per ml to 1.2×10^7 cells per ml in the case of succinate. This was correlated to a decrease in the percentage of nitrification compared to the control culture. However, the number of nitrifying cells remained constant (10^6 cells per ml). In the control culture, there was 99% of ammonium oxidation in nitrite which corresponded to 83.8 mg N-oxide produced $l^{-1}.d^{-1}$ and 1% of assimilation for protein synthesis (6 $mg.l^{-1}$). In the presence of carbon, the overall quantity of ammonium consumed decreased and the part oxidized in N-oxides decreased too, on behalf of the part used for protein synthesis. For example, with ethanol or succinate, the increase of protein synthesis (188 and 147 $mg.l^{-1}$, respectively) was concomitant to the decrease in N-oxides production: 56.6 and 68.3 $mg.l^{-1}.d^{-1}$. On the other hand, a lower consumption of the carbon source implied little changes in nitrifying activity. This was the case in the presence of caproate where 99% of ammonium was oxidized to nitrite. Addition of yeast extract resulted in smaller protein synthesis compared to that observed with ethanol, but with no

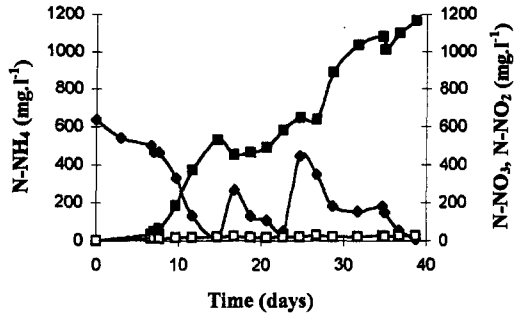

Fig. 1. Evolution of ammonium (◆), nitrate (□) and nitrite (■) concentrations in the control flask during batch culture of the autotrophic nitrifying microflora.

perturbation of the nitrifying activity. This might be explained by the consumption of yeast extract directly as nitrogen and energy sources, evading competition for ammonium between heterotrophs and autotrophs.

Despite the highest affinity for ammonium of autotrophs, compared to heterotrophs, the nitrifying activity decreased in the presence of a carbon source, because of higher heterotrophic cell numbers and competition for oxygen and ammonium. As a conclusion, the quantity and the nature of the carbon source are determinant factors for maintaining heterotrophic growth and activity with a simultaneous nitrifying activity.

Influence of combined addition of carbon source and of the strain SGLY2 in the batch cultures

In the following experiments, the carbon source was added discontinuously (once a day) to the medium, to reach a final concentration of 250 $mg.l^{-1}$. Two concentrations of supply of the strain SGLY2 were tested. Addition of the strain SGLY2, without carbon source, in the nitrifying culture had no effect on the nitrifying activity. The strain, without any electron donor, was unable to denitrify and to grow. The simultaneous addition of the aerobic denitrifier and of the carbon source had different consequences, depending on the nature of this carbon source and of the quantity of cells inoculated. Evolution of the N-oxides produced in the control flask is shown on Fig. 1. There was an equivalence between ammonium consumption, nitrite and biomass accumulation. On the other hand, evolution of the N-oxides in test Erlenmeyer flasks was quite different (Fig. 2). In fact, whereas nitrite accumulated in the control flask (discontinuous culture), the N-oxides concentration levelled off or fell for the different carbon sources. Table 1 summarizes the nitrogen balances for each combined addition of carbon and of the pure

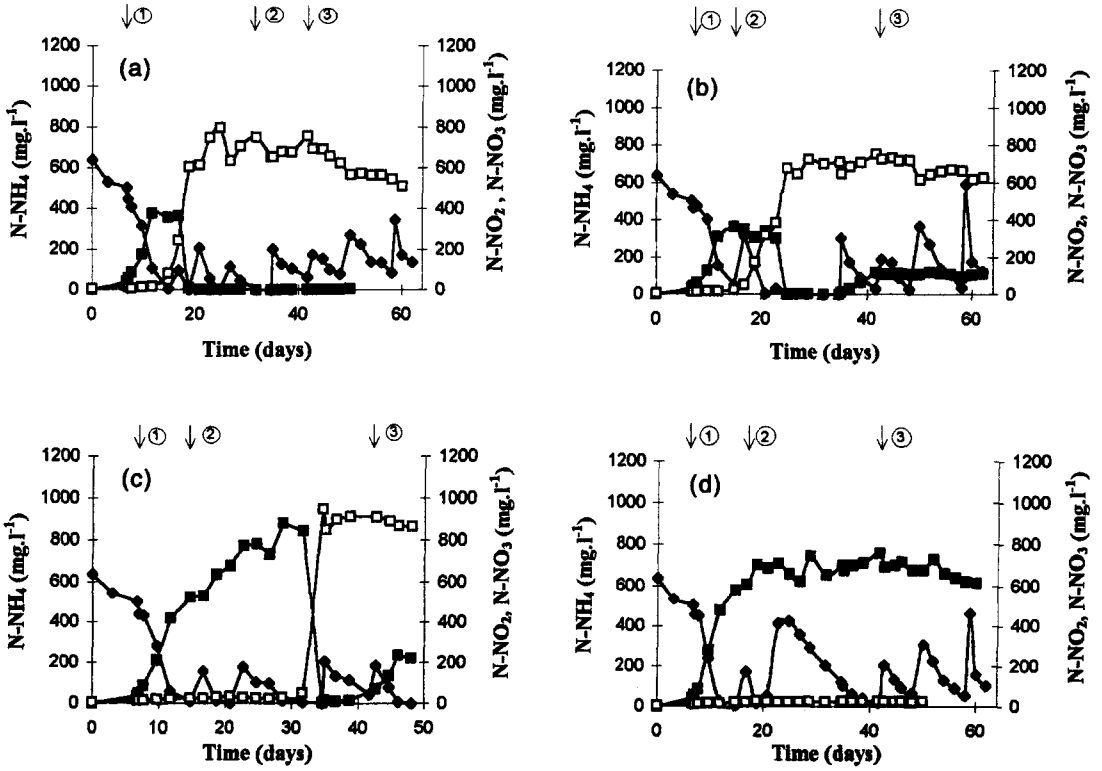

Fig. 2. Effect of combined supply of carbon source and of strain SGLY2 on evolution of ammonium (◆), nitrate (□) and nitrite (■) concentrations during aerated batch culture. (a) presence of succinate; (b) presence of ethanol; (c) presence of caproate; (d) presence of yeast extract; ① addition of carbon source; ② first supply of carbon source and of strain SGLY2; ③ second supply of carbon source and of strain SGLY2.

Table 1. Nitrogen balances for each supply of carbon source and of strain SGLY2 in batch co-culture

Carbon source	Period	$N\text{-}NH_4^+$ consumed ($mg.l^{-1}.d^{-1}$)	$N\text{-}NO_x$ produced ($mg.l^{-1}.d^{-1}$)	N-protein ($mg.l^{-1}.d^{-1}$) (and %)	ΔN ($mg.l^{-1}.d^{-1}$)
Ethanol	(2)	79.8	38	10.2 (12.8)	31.5
	(3)	26.8	0	7.2 (27)	19.5
Succinate	(0)	73.2	68.3	5 (7)	0
	(2)	23.3	17	1.3 (5.4)	5
	(3)	16.3	0	5.4 (33)	10.9
Caproate	(2)	73.5	55.9	2.1 (2.8)	15.5
	(3)	31.3	17.3	1.8 (5.7)	22
Yeast extract	(2)	78.4	49.5	6.9 (8.7)	22
	(3)	22.6	0	7.4 (32)	15.3

(0) before supply of strain SGLY2 and of carbon source.
(2) first supply of carbon source and of strain SGLY2.
(3) second supply of carbon source and of strain SGLY2.

strain, considering that there was no nitrogen stripping.

The first simultaneous addition with a protein ratio of SGLY2/nitrifiers = 2 resulted in an increase of the protein synthesis. Consequently, between 3 and 12% of ammonium, depending on the carbon source, was consumed for cellular growth. The remaining quantity was oxidized in N-oxides. According to the results of spreading on Petri dishes, the strain SGLY2 seemed to be present but with a decreasing cellular concentration with time. At the second simultaneous addition (SGLY2/nitrifiers = 20), the part of ammonium assimilated increased for the best metabolized carbon sources, with 27 and 33% of assimilation for ethanol and succinate, respectively. With a worse metabolized carbon source, caproate, the percentage of assimilation was lower (6%). This second phase was also accompanied by a slow-down in ammonium consumption rate.

Introduction of the strain SGLY2 in the nitrifying culture ended up to with a nitrogen imbalance: there was less N-oxides accumulated than ammonium disappeared. Sometimes, the ammonium oxidation products were not found in the medium, even with caproate, the worse metabolized carbon source. The N-oxides disappearance could only be explained by aerobic reduction of the nitrogen compounds by the strain SGLY2. It is possible to calculate the proportion of N-oxides produced that were reduced by the aerobic denitrifier (Table 2). In the case of ethanol, succinate and yeast extract, 100% of denitrification can be calculated: that is an immediate consumption of the N-oxides produced by autotrophic nitrifiers.

Table 2. Percentages of denitrification for each supply of strain SGLY2 depending on the carbon source in batch co-culture

Carbon source	Period	% of $N\text{-}NO_x$ reduced
Ethanol	(2)	46
	(3)	100
Succinate	(0)	0
	(2)	23
	(3)	100
Caproate	(2)	21
	(3)	75
Yeast extract	(2)	30
	(3)	100

In particular conditions of massive inoculation of SGLY2 and of fractionated supply of carbon, it was possible to observe simultaneous nitrification and denitrification, whatever the intermediate products of nitrification. The strain SGLY2 seemed to be dominant compared to the other heterotrophs (6×10^6 cells per ml on 1.5×10^8 cells per ml), to maintain itself and to be active.

These experiments showed the feasibility of association of a complex nitrifying microflora with an aerobic denitrifier in a single aerated system. However, in these batch cultures, there were no direct measurements of dissolved oxygen concentration and of gas production. Moreover, there was no pH control. For these reasons, a continuous culture was carried out in a 2 l Biolafitte reactor.

Combined nitrification and denitrification in continuous culture

The continuous culture started with an ammonium load of 44.6 $N\text{-}NH_4^+$ $mg.l^{-1}.d^{-1}$ with a constant dilution rate of $0.336\ d^{-1}$. After complete stabilization of the nitrifying population, which was characterized by a stable biomass concentration ($10\ mg.l^{-1}$), the percentage of nitrification was 63.2% with a nitrification rate of 28.2 $N\text{-}NO_x$ $mg.l^{-1}.d^{-1}$. This implied that a part of ammonium is rejected in the effluent.

The first test corresponded to continuous supply of C-succinate ($500\ mg.l^{-1}$) by the feeding medium. This resulted in a complete disequilibrium of the system with an increase of the biomass concentration up to $85\ mg.l^{-1}$. Consequently, at steady state, there was an increase of the percentage of assimilation (18%) and of residual ammonium (76%), and a decrease of the percentage of nitrification to 5%. This implied that a continuous carbon supply favoured the heterotrophs growth despite the nitrifying microflora because of a higher growth rate and of competition for ammonium. There was no competition for oxygen because this factor was maintained at a non-limiting

Table 3. Nitrogen balances for each combined supply of carbon source and of strain SGLY2 under continuous aerated mixed culture. For calculation, see Materials and Methods. $A = [N\text{-}NH_4]_{j-1} \cdot Q/V$ ($mg.l^{-1}d^{-1}$); B and C in $mg.l^{-1}d^{-1}$

Experiment	A	B	C	Balance $A-B-C$	Nitrogen gas
1	44.5	42.7	−10.9	12.6	N_2
	44.5	41.9	5	−2.5	—
2	44.5	40.5	−23.8	27.8	N_2
	46.3	48.2	−30.5	28.6	N_2
3	41.6	37.7	−4.4	8.1	N_2
	41.6	37.9	5.1	−1.4	—
4	37.4	27.5	−5.6	15.5	$N_2O + N_2$
	37.4	31.9	−5.3	10.7	$N_2O + N_2$
	37.4	33.8	12.8	−9.2	$N_2O + N_2$

concentration. This result, combined to those obtained in batch cultures, led to the same conclusion: to maintain a good nitrifying activity and to restrict the growth of heterotrophs, the carbon source has to be supplied discontinuously. It was supplied every day at the same hour.

Before starting the different tests, another steady state under strictly nitrifying conditions was reached with 80% of nitrification (90% nitrate:10% nitrite) and a residual ammonium concentration of 39 $mg.l^{-1}$. The different tests corresponded to the combined supply of the strain SGLY2 and succinate at various concentrations. These tests lasted 1 week, that is twice the retention time, which was enough to reach steady state. For each experiment, a nitrogen balance was established on several dates in the week (Table 3). These balances were calculated on the mass of components between 2 days. A positive balance showed that there was less assimilation, $N\text{-}NO_x$ production and nitrogen loss than supply of ammonium. There was, then, a loss of ammonium that could be explained by an underestimation of the $N\text{–}NO_x$ production because of a simultaneous production–consumption by autotrophs and the strain SGLY2, respectively. In this case, a distinction was made between apparent and effective nitrification which allowed us to calculate a denitrifying percentage.

During these weeks, the biomass concentration fluctuated due to the discontinuous supply of carbon: there was a decrease in the global protein concentration and modification of the constituents of this biomass. Consequently, just after each supply, there was a decrease in $N\text{-}NO_x$ concentration in the effluent, followed by an increase corresponding to the disappearance of the aerobic denitrifier (decrease of the number of colonies on nitrate–succinate agar plates). These observations had to be correlated with the positive balance observed in the earlier days of each experiment (Table 3) and the negative or null balance observed in the latter days. This implied that, immediately after addition of the pure strain and of succinate, the aerobic denitrifier was active and there was simultaneous consumption of the N-oxides produced by nitrification. The main problem was to maintain the heterotrophic biomass, particularly the strain SGLY2. That is why we tested various concentrations.

The first and third supply of the aerobic denitrifier (final concentration 25 and 50 $mg.l^{-1}$, respectively) and succinate (final concentration in C-succinate 13 and 50 $mg.l^{-1}$, respectively) had a gentle effect on apparent nitrifying activity because of little modification of the percentage of nitrification compared to that of the strictly nitrifying control condition (Table 4). There was a higher ammonium consumption for protein synthesis of the heterotrophic population (12.5 and 6.4%, respectively), corresponding to a decrease in residual ammonium concentration (16–10 $mg.l^{-1}$). However, the concentration of global heterotrophic biomass decreased with time because of the small supply of carbon. The percentages calculated at the end of the first supply correlated this fact: there was a smaller assimilation percentage (2.7%) with a higher residual ammonium percentage (30.5%). The balance was then quite equilibrated with an error smaller than 5%.

In the first case, the concentration of added carbon was not enough to maintain the heterotrophic biomass. To counter this, during the third supply at the end, the biomass maintained itself, but the proportion of aerobic denitrifier decreased, implying loss of denitrifying activity.

In the two cases, a positive balance was well correlated with the presence of nitrogen in the gaseous phase and with calculation of denitrifying percentages of 7 and 21%. This second higher figure could be explained by the higher concentration of added SGLY2. However, at the end, the strain SGLY2 was diluted with time in the heterotrophic biomass.

In the second and fourth supply, the final concentration of the strain SGLY2 was 40 and 50 $mg.l^{-1}$, respectively, and the final concentration of succinate was 50 $mg.l^{-1}$. These supplies corresponded to: (i) a decrease in the percentage of apparent nitrification (33 and 20%, respectively) compared to the previous test; (ii) a high disequilibrated nitrogen balance; and (iii) presence of nitrogen and nitrous oxide in the gaseous phase. These facts implied that a part of the N-oxides produced was presumably consumed by the heterotrophs and especially by the

Table 4. Percentages of apparent nitrification (AN), of effective nitrification (EN), of assimilation (AS), of residual ammonium (RA) and of denitrification (D) for each experiment during continuous aerated mixed culture

Experiment	AN	AS	RA	EN	D
1	80	12.5	2	86	7
	67.3	2.7	30.5	—	—
2	35	2	0	97.9	64
	32	5.6	0	96.4	66.8
3	70.9	6.46	2.9	90.56	21.7
	86.8	7.8	5.2	—	—
4	20.8	18.2	19.4	62.3	66.6
	33.8	7	30.3	57.6	41.3
	42.6	11.8	45.6	—	—

strain SGLY2. The percentage of denitrification was estimated around 65% for the two supplies.

The difference between the two supplies was the percentage of residual ammonium. During the second supply, the overall quantity of ammonium was consumed with a concurrently higher percentage of effective nitrification (96%). To counter this, during the fourth supply, the percentage of effective nitrification was smaller (60%), with higher percentages of residual ammonium and of assimilation.

After these 4 weeks of carbon supply, the system was quite disequilibrated with a decrease in activity of the autotroph nitrifying biomass, and an increase in protein synthesis and in loss of ammonia. Therefore, the strain SGLY2 seemed to maintain itself during more days than in the first experiment and to reduce the N-oxides produced by nitrifiers under aerobic conditions (dissolved oxygen concentration: 14 $mg.l^{-1}$).

The higher the quantities of aerobic denitrifiers and of carbon source, the smaller is the percentage of apparent nitrification, but the higher is the percentage of denitrification. However, the loss in nitrifying activity at the end of the fourth supply was not only due to an increase of assimilation, but was a consequence of an increase of the residual ammonium concentration. In fact, the fractionated supply of carbon limited the biomass growth, evading competition for ammonium, and allowed activity of the two populations of nitrifiers and denitrifiers. These results confirmed the facts observed in the discontinuous cultures and showed over again the possibility of coupling nitrification and denitrification in a single aerated reactor.

DISCUSSION

Existence of a mixed ecosystem able to nitrify, denitrify and consume organic matter in a single-stage aerobic system would be an attractive and revolutionary idea in the world of wastewater treatment processes. Therefore, this kind of culture may exhibit a variety of interactions that can be either detrimental or beneficial for the organisms involved. The first objective is to find the environmental factors that would maintain in balance the different microflora. Discontinuous and continuous cultures, described above, constitute a new approach to the conception of nitrogen removal by association of a complex nitrifying microflora and of an aerobic denitrifier (pure culture). In this system, interactions are various and complex implying various performances for the reactor depending on the conditions applied. Many things are known about the effect of pH, temperature, dissolved oxygen concentration and carbon source on the different populations considered independently. For example, addition of increased concentrations of glucose in soil columns resulted in a decrease of ammonium-oxidizing activities although 10^7 nitrifying cells per g of dry soil were still present (Verhagen *et al.*, 1992). It might be explained by a decrease of the autotrophs' affinity for ammonium through competition with a high number of heterotrophic cells. In counter part, several papers reported on the stimulation of autotrophs due to excretion of organic products by heterotrophs (Blanc *et al.*, 1986). Another example is the stimulation of *Nitrobacter agilis* growth by nitrite produced by a heterotrophic nitrifier from pyruvic oxie (Castignetti and Gunner, 1980).

In our experiment, competition occurred between two populations which catalyse different reactions in the nitrogen cycle, but compete for common substrates: competition for oxygen and ammonium between autotrophs and heterotrophs, and competition for oxygen, ammonium and carbon between heterotrophs and the strain SGLY2. The competition for oxygen was overcome through the choice of the working dissolved oxygen concentration which was non-limiting. It was found that the availability of organic carbon source, relative to the available amount of ammonium, and the concentration of biomass supplied were key factors in the outcome of such competition. At low biomass concentration, the strain SGLY2 did not maintain itself, with consequently no denitrifying activity. In counter part, at high biomass concentration, its higher ammonium consumption resulted in an inbalance between the percentages of nitrification and assimilation. By limiting the carbon supply, we found a balance between nitrifying, assimilating and denitrifying activities. However, the major problem was that the discontinuous supply of carbon limited the heterotrophic biomass without distinction between the pure strain of interest and the other heterotrophs. If the protein concentration levelled off after 1 week of supply, the ratio between the strain SGLY2 and the heterotrophs evolved with less and less pure strain SGLY2. To allow maintenance of the strain in the reactor, a fixed support could be used such as agar beads. However, these ratio calculations were based on the determination of the number of colonies on nitrate–succinate agar plates incubated under anoxic conditions. The morphological observations showed that this number was overestimated. This kind of biomass follow-up was not specific enough and has to be improved. Other methods exist that are based on the use of fluorescently labelled 16S rRNA-targeted oligonucletide probes in combination with an epifluorescence microscope for *in situ* monitoring of bacteria (Wagner *et al.*, 1994).

Similar studies were carried out in mixed continuous culture of a heterotrophic nitrifier/aerobic denitrifier, *Thiosphaera pantotropha*, and an autotrophic nitrifier, *Nitrosomonas europaea*, (Van Niel *et al.*, 1993). They observed that, at a high C-acetate/N-ammonium ratio, *Thiosphaera pantotropha* was able to outcompete *Nitrosomonas europaea* for ammonium: 44% of ammonium was

consumed for biomass synthesis at C/N = 10. With increasing C/N ratios, the part of residual ammonium increased too from 0% at C/N = 2 to 15% at C/N = 10. The quantity of nitrite produced decreased too with an increase of the part of ammonium transformed to gas. Moreover, they showed that at a cellular ratio *Thiosphaera*/*Nitrosomonas* superior to 260, *Thiosphaera pantotropha* became dominant, by competing with *Nitrosomonas europaea* directly for ammonium oxidation. At this point, the behaviour of the strain SGLY2 differs from that of *Thiosphaera pantotropha* because our strain is unable to make use of heterotrophic nitrification. However, these results underline that a balance has to be found between maintenance of biomass concentration and regulation of activities of the present biomass. The important factor is to inoculate the complex medium with a high concentration of the pure strain. Another idea could be the fixation of the biomass on a support.

Another experiment was carried out with *Thiosphaera pantotropha* mixed with an activated sludge (Kshirsagar *et al.*, 1995). In a continuous stirred aerated factor (dissolved oxygen concentration of 2.4 $mg.l^{-1}$), 80% of nitrate was reduced compared to 16–28% of reduction in the control reactor without the strain. It is then possible to maintain an aerobic denitrifier in a complex heterotrophic microflora and to observe aerobic nitrate reduction. These results lie close to those obtained with the strain SGLY2. They all show the feasibility of maintaining an heterotrophic aerobic denitrifier in a mixed population of heterotrophs and autotrophs and of creating a single-stage aerobic reactor where nitrification and denitrification could take place simultaneously.

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