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COMBINED PHOSPHATE AND NITROGEN REMOVAL IN A SEQUENCING BATCH REACTOR USING THE AEROBIC DENITRIFIER, *MICROVIRGULA AERODENITRIFICANS*

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Abstract—A phosphate removal sludge was bioaugmented with the aerobic denitrifier, *Microvirgula aerodenitrificans* in order to reduce the nitrate produced during the aerobic nitrifying-phosphate uptake phase. Fluorescent *in situ* hybridization (FISH) was used to follow the fate of the added strain. In order to maintain the pure strain in the complex ecosystem, diverse physiological and kinetic based strategies of bioaugmentation were tested under the sequencing batch reactor (SBR) type culture. The nature of the *M. aerodenitrificans* inoculum (adapted to nitrate–aerobic conditions or to anoxic one) had no influence on the SBR performances and did not enhance aerobic denitrifying performances. The optimum quantity of the added strain (10% of the total biomass) seemed to have much more positive influence on the long term maintenance of the pure strain than on the SBR performances. A small but daily supply of *M. aerodenitrificans* gave exactly the same result than a massive and 1-day supply, i.e. no enhancement of performances and no amelioration of the length of maintenance. A continuous supply of carbon during the first hour of the aerobic phase combined to a 10% supply of *M. aerodenitrificans* gave the best compromise in terms of phosphate removal, nitrification and aerobic denitrification performances. It was accompanied too by a decreased number of the ammonia and nitrite-oxidizing bacteria and a modification of the nitrite-oxidizing floc structure. FISH on *M. aerodenitrificans* revealed that (i) before bioaugmentation, the strain was already present in the phosphate removal sludge and (ii) the added bacteria almost disappeared from the reactor after 16 HRT. In a last experiment, *M. aerodenitrificans* embedded in alginate beads allowed enhancement of both aerobic denitrifying performances and length of strain maintenance.

Key words—phosphate removal, nitrogen removal, aerobic denitrification, *Microvirgula aerodenitrificans*, fluorescent *in situ* hybridization, bioaugmentation

INTRODUCTION

Removal of nitrogen and phosphate from wastewater is becoming a more and more acute problem for municipalities and industries as effluent quality standards become more stringent. Indeed, the European directive of 21 May 1991 has defined for sensitive areas (1) a maximum total nitrogen and phosphorus concentration in released wastewater of 15 and 2 mg/l for sites with 10,000–100,000 population equivalent (PE), of 10 and 1 mg/l for sites bigger than 100,000 PE, (2) a yield higher than 70 and 80%. However, the actual average yield for nitrogen and phosphorus treatment of urban wastewater surrounds 40%. This last figure underlines the importance of enhancement of existing processes.

Conventional nitrogen removal plants are based on the combination of nitrification (aerobic oxidation of ammonia into nitrate) and denitrification (anoxic conversion of nitrate into nitrogen gas). In terms of reactor configuration, this implies spatial or temporal separation of the two phases.

Up to date, phosphate removal is realized either by chemical precipitation, or by phosphate incorporation into biomass. Enhanced biological phosphate removal (EBPR) systems can be considered as an effective alternative to chemical precipitation because of its low investment, low operational costs and better quality of sludge (Lan *et al.*, 1983). An anaerobic/aerobic sequence is necessary to promote biological phosphate removal: a P release in the anaerobic stage followed by an excess of P uptake in the aerobic stage, P accumulation as poly-phos-

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phates being bigger than P release. Another important point is that readily bio-assimilable compounds have to be present during the anaerobic step. Many observations on these cyclically systems show that during the aerobic phase, ammonia is oxidized into nitrate by a nitrifying indigenous population. The integrated configurations proposed are then constituted of three steps: anaerobic, aerobic and anoxic in order to obtain an effluent exempted of phosphate and nitrate (Cooper *et al*., 1994).

However, the discovery of atypical behaviors of nitrogen fixing, nitrifying, ammonifying and denitrifying bacteria (Jetten *et al*., 1999) may allow to propose new integrated phosphate and nitrogen removal systems. Indeed, many studies reported existence of bacteria able to reduce N-oxides aerobically (Robertson *et al*., 1989; Bonin and Gilewicz, 1991; Patureau *et al*., 1994; Carter *et al*., 1995; Frette *et al*., 1997; Lukow and Diekmann, 1997). For example, Kshirsagar *et al*. (1995) demonstrated the feasibility of combining nitrification and denitrification in a single aerobic reactor by inoculating activated nitrifying sludge with *Thiosphaera pantotropha*, a well known aerobic denitrifier. Another well known aerobic denitrifier, named *Microvirgula aerodenitrificans* (Patureau *et al*., 1998), was also used to inoculate continuous (Patureau *et al*., 1997) and sequencing batch (Bouchez *et al*., 2000a) nitrifying aerobic reactors in order to obtain a complete nitrogen removal. It implies thus the elimination of the anoxic phase in the previous integrated phosphate and nitrogen removal configuration. The experiments with *M. aerodenitrificans* show that it is possible to obtain simultaneous nitrification and denitrification but with yields depending on the strategies of bioaugmentation. These latter have to be more defined and optimized to be sure to maintain the strain or to spread and activate the aerobic denitrifying function. The aim of this paper was to study an integrated process for phosphate and nitrogen removal fed with a synthetic urban wastewater. The system consisted in a sequencing batch reactor with anaerobic and aerobic phases bioaugmented with *M. aerodenitrificans*. The effect of the nature of the added cells, of amount and of supply frequency on bioreactor performances and on bioaugmentation efficacy were tested. Indeed, these factors are thought to be important parameters which condition the success of bioaugmentation (Ramadan *et al*., 1990; Kennedy *et al*., 1990; Comeau *et al*., 1993). The fate of the added strain was monitored both by follow up kinetic parameters and by fluorescent *in situ* hybridization (FISH).

MATERIALS AND METHODS

Microorganisms

The strain *M. aerodenitrificans* (BCCM/LMG 18919) used in this study was isolated from an upflow filter where aerating conditions were fluctuated (Patureau *et al*., 1998).

Inoculation sludge for the SBR was obtained from a phosphorus and nitrogen removal process operating on urban wastewaters at Roanne (France).

Media and growth conditions

The composition of the synthetic wastewater and of the oligo-element solution is described in Kuba *et al*. (1993). During the experiment, the wastewater was maintained at 4°C. The strain *M. aerodenitrificans* was cultivated under agitation (250 rpm), at 35°C and pH 7 under aerobic (Erlenmeyer) as well as anoxic (sealed flask) conditions in a synthetic medium described in Patureau *et al*. (1994). These pre-cultures were used to inoculate the SBRs. The initial culture volume depended on the quantity of biomass needed for bioaugmentation. *M. aerodenitrificans* was also embedded in high-strength gel alginate beads according to the following conditions: 0.33 g dry mass of *M. aerodenitrificans* was suspended in 20 ml 0.1 M KCl and mixed to 100 ml of a 4% sodium alginate solution with low M/G content (Manugel DMB, Kelco Nutrasweet). The obtained mixture was pumped and extruded through a hollow needle into a 0.1 M $CaCl_2$ cross-linking solution. The resulting beads (2.5–3.5 mm diameter) were cured in the gelling solution for 1 h (Bouchez *et al*., 2000b).

Process configuration

The study was carried out in a 2 l Biolafitte reactor (1.5 l of working volume). The reactor content was continuously agitated at 250 rpm by impellers except in the settling period. The SBR was operated in a cycle of 6 h. The cycle consisted of five phases: idle (0.75 l of influent during 2 min), anaerobic period (2.5 h), aerobic period (2.5 h), settling period (0.75 h) and drawing (0.75 l of clarified supernatant during 10 min). The hydraulic retention time (HRT) was 12 h. The sludge retention time (SRT) was 52 days with a constant VSS of 4 g/l. The sludge age was controlled by sampling of mixed liquor for cycles analysis (2 analyzed cycles per week). pH and temperature were maintained, respectively, at 7.5 (HCl 0.5 N) and 20°C. Each experiment was realized during 30 HRT (15 days).

Whole cell fluorescent in situ hybridization

Fixation of cells with paraformaldehyde 4% was performed as described by Manz *et al*. (1992) except that the centrifugation step was replaced by filtration on a 0.2 μm nylon filter (Gelman sciences). Whole cell *in situ* hybridization were performed under stringent conditions as described previously (Manz *et al*., 1992). The fluorescent rRNA targeted probes are fully detailed in Bouchez *et al*. (2000a). The probe S-D-Bact-0338-a-A-18 (Eub-338) was used as a positive control. Two probes S-S-Mae-1414-a-A-18 and S-S-Mae-0829-a-A-18, specific for *M. aerodenitrificans*, are used in order to be sure to detect specifically the aerobic denitrifier. The optimal hybridization stringency for these two oligonucleotide probes was determined by quantification of the fluorescence conferred by the probe to target and non-target reference cells at different formamide concentrations in the hybridization buffer as outlined by Manz *et al*. (1992). Montage and observation of samples are well described in Bouchez *et al*. (2000a). For each measurement at least 300 cells were analyzed for each hybridization condition, the mean values of triplicate measurements were determined.

Analytical methods

Samples were centrifuged twice at 15,000 rpm (4°C, 10 min) and then analyzed for nitrate, nitrite, phosphate, ammonia, acetate as described elsewhere (Rustrian *et al*., 1997). Volatile suspended solids (VSS) was calculated after

centrifugation of 10 ml of sample according to standard methods (APHA, AWWA, WPFC, 1985).

Calculations

Phosphate removal yield (Yp) on a cycle:

$$Yp = [P - PO_4^{3-}]_{acc}/F \times [P - PO_4^{3-}]_{in}$$

$$[P - PO_4^{3-}]_{acc} = [P - PO_4^{3-}]_{si} - [P - PO_4^{3-}]_{ei}$$

$$[P - PO_4^{3-}]_{si} = F \times [P - PO_4^{3-}]_{in} + (1 - F) \times [P - PO_4^{3-}]_{e(i-1)}$$

$[P-PO_4^{3-}]_{in}$: phosphate concentration in the influent; $[P-PO_4^{3-}]_{si}$: phosphate concentration at the start of cycle i; $[P-PO_4^{3-}]_{ei}$: phosphate concentration at the end of cycle i; and F: dilution factor of the influent in reactor

Apparent denitrification (ΔN) on a cycle was expressed as:

$$\Delta N = ([N - NH_4^+]_{si} + [N\text{-}NO_x^-]_{si}) - ([N - NH_4^+]_{ei} + [N\text{-}NO_x^-]_{ei}) - [\text{N-assimilation}]$$

$[N]_{si}$: concentration of nitrogen at the start of cycle i; $[N]_{ei}$: concentration of nitrogen at the end of cycle i;

$$[N\text{-}NO_x^-] = [N\text{-}NO_3^-] + [N\text{-}NO_2^-]$$

[N-assimilation] was determined through the measurement of VSS produced during one cycle, considering that 12% of the biomass is nitrogen (McCarty, 1969). Average values were calculated on 9, 11 and 7 cycles for, respectively, control cycles, acetate tests and bioaugmentation experiments.

Percentage of N-oxides: %N-oxides

$$= ([N\text{-}NO_x^-]_{ei} - [N\text{-}NO_x^-]_{si})/[N - NH_4^+]_{si} \times 100$$

Percentage of residual ammonia: $\%N - NH_4$

$$= ([N\text{-}NH_4^+]_{ei}/[N -^+ NH_4]_{si}) \times 100$$

Percentage of nitrogen assimilated: %N-assimilation

$$= ([\text{N-assimilation}]/[N - NH_4^+]_{si}) \times 100$$

Percentage of apparent denitrification: $\%\Delta N$

$$= (\Delta N/[N - NH_4^+]_{si}) \times 100$$

Percentage of anoxic denitrification: $\%\Delta N_{anoxic}$

$$= (([N\text{–}NO_x^-]_{si} - [N\text{-}NO_x^-]_{e\text{-}anaero\text{-}i})/[N - NH_4^+]_{si}) \times 100$$

$[N]_{e\text{-}anaero\text{-}i}$: concentration of nitrogen at the end of the anaerobic period of the cycle i

Percentage of aerobic denitrification: $\%\Delta N_{aerobic}$

$$= \%\Delta N - \%\Delta N_{anoxic}$$

RESULTS

Study of control cycles

Evolution of phosphate, nitrate, nitrite and ammonium during one control cycle is presented on the Fig. 1. In terms of phosphate removal, an average yield of 96% was calculated on 10 cycles, that is five HRT. The phosphorus concentration in the effluent varied between 0 and 2 mg/l. Ammonia was entirely consumed. However, only 66% (average

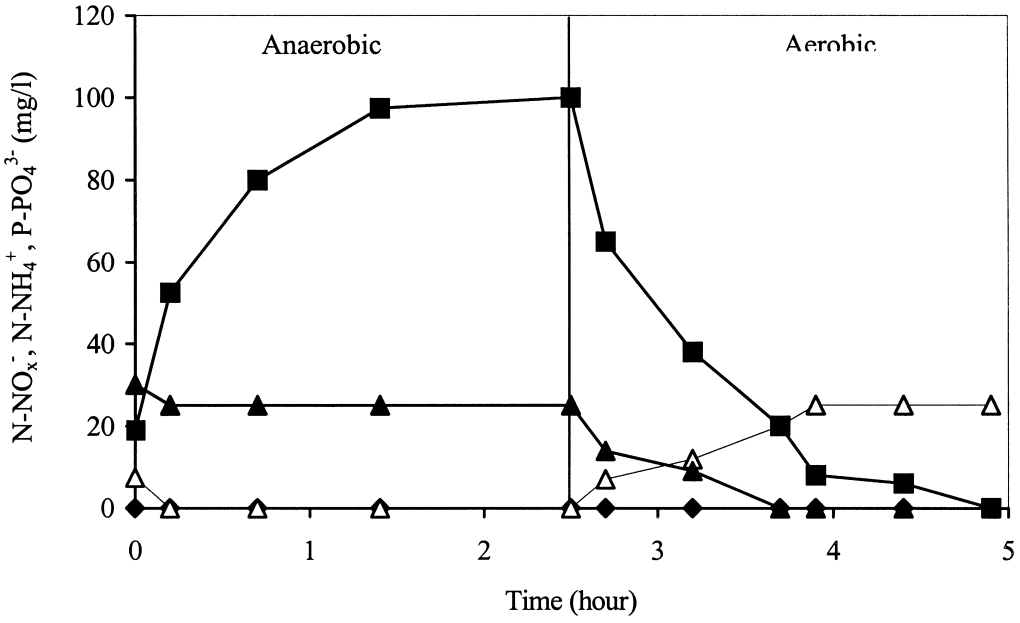

Fig. 1. Phosphate, nitrate and ammonium profiles for one cycle of a well adapted phosphate removal SBR. Δ $N\text{-}NO_3^-$, ◆ $N\text{-}NO_2^-$, ■ $P\text{-}PO_4^{3-}$, ▲ $N\text{-}NH_4^+$ (mg/l).

percentage on the 10 cycles) of the initial ammonia concentration was oxidized into N-oxides, principally nitrate (20 mg/l). The latter parts were: 14% for assimilation, 15% for anoxic denitrification. A 5% loss in the nitrogen balance was noticed during the aerobic phase (Table 1). Thus, the effluent is enriched in nitrate and exempted of phosphate and ammonium.

In situ hybridization on the native sludge revealed that the nitrifying population was mostly composed of bacteria present in the flocculated form and that belong to the β subclass of the *Proteobacteria* group. Hybridization with S-S-Mae-1414-a-A-18 revealed that *M. aerodenitrificans* was present in the native sludge at a low level. In order to be sure of this result, another probe was then used to validate results obtained with S-S-Mae-1414-a-A-18. The crossed hybridization on native sludge showed the presence of *M. aerodenitrificans* too.

Influence of different strategies of carbon supply for aerobic denitrification on nitrogen and phosphorus removal performances

Before bioaugmentation, the effect of the addition of carbon on phosphate removal performances was tested. Indeed, *M. aerodenitrificans* as an heterotrophic denitrifier needs to consume carbon to denitrify and will compete for carbon with the phosphate removing bacteria during the anaerobic phase and with heterotrophs during the aerobic phase. Since acetate is commonly used in wastewater treatment and since it is consumed by *M. aerodenitrificans*, it was chosen as electron donor for aerobic denitrification. As a first strategy, a punctual and massive supply was realized at every cycle, 1 h after the beginning of the aerobic phase. A 180 mg of C-acetate supply (quantity in the reactor) led to a phosphate release during the aerobic phase, implying a released waste with nutrient concentration superior to the standards. At lower supplies (60 mg), no phosphate release during the aerobic phase was observed. In contrast, the P-release and P-uptake profile was completely modified with a Yp of 30.5% (average on 10 HRT). Moreover, nitrification was completely disturbed with production of nitrite, production of biomass and residual ammonia in the effluent.

Table 1. Nitrogen balance during control cycles (average on 10 cycles) and test with acetate (average on 12 cycles). During the test with acetate, the carbon source was continuously supplied during the first hour of the aerobic phase in order to have a resulting quantity of 60 mg of C-acetate

	Control cycles	Tests with acetate
%N-oxides	66	52
%N-NH_4	0	0
%N-assimilation	14	31
$\%\Delta N_{anoxic}$	15	4
$\%\Delta N_{aerobic}$	5	13

Table 2. Effect of nature of pre-culture, amount and supply frequency of *M. aerodenitrificans* on nitrogen performances. The size of inoculum was expressed as a percentage amongst the total biomass. Each experiment was realized independently. Before bioaugmentation tests, a characteristic kinetic phosphate removal profile was obtained like for the control cycles. The zero HRT corresponded to the day of concomitant acetate and *M. aerodenitrificans* additions

Nature of pre-culture	Anoxic						Aerobic											
Frequence of inoculum	One massive addition			One massive addition			One massive addition			One massive addition			Daily supply					
Size of inoculum (%)	3.7			3.2			10.3			15			1.6			1% per day		
HRT (day)	0	8	22	0	6	14	0	6	14	20	32	0	4	8	22	0	4	8	16	42	56	60
%N-oxides	48	37	60	56	34	70	60	41	48	49	56	98	56	53	57	82	59	48	50	40	42	46
%N-NH_4^+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
%N-assimilation	24	25	35	23	24	26	33	25	23	26	37	37	25	25	21	32	25	25	23	25	25	23	25
$\%\Delta N_{anoxic}$	9	5	0	0	0	4	0	14	0	10	0	5	25	11	9	15	12	12	12	12	9	15	14
$\%\Delta N_{aerobic}$	19	33	5	21	42	0	7	0	35	15	14	2	−48	8	17	−4	−19	4	17	13	26	20	15

These two massive supplies had then induced the growth of heterotrophs to the detriment of nitrifiers and phosphate removing bacteria. Other modalities of supply were tested during the aerobic phase, considering that a continuous supply would be more adequate. Indeed, a high affinity constant for acetate was calculated for *M. aerodenitrificans* through model establishment (personal communication). A continuous supply during the first hour of the aerobic phase (with a resulting C-acetate supply of 60 mg) gave the best results with an average percentage of phosphate removal of 100% on 6 HRT. Ammonia was completely consumed with a nitrate production of 52% (Table 1). This carbon supply implied too an increase in the quantity of biomass produced, leading to an increase of the N-assimilation part (31%). In the same time, the nitrogen fraction that disappeared during aerobic phase doubled. Whereas no perturbation of phosphate removal activity was observed, it seems that addition of acetate favored growth of heterotrophs and slowed down the nitrifying activity because of ammonium competition. This can be correlated to FISH results showing a decrease in the nitrifying community.

Influence of diverse strategies of inoculation of M. aerodenitrificans on SBR performances

Three tests were realized in order to optimize the inoculation conditions through the nature and quantity of added cells of *M. aerodenitrificans* and frequency of supply. Concomitantly to the addition of cells, acetate was added and consumed during the first hour of the aerobic step. The quantity added was equal to 60 mg (C-acetate). The first observation was that phosphate removal performances were never affected by the inoculation strategy and varied between 96 and 100%. In contrast, the nitrogen balance was completely modified.

Cells used to inoculate the SBR were first adapted to anoxic or aerobic conditions in presence of nitrate, the quantity added being constant (3.7 and 3.2%, respectively, expressed in percentage of the reactor biomass). Calculations showed that at the day of inoculation and 6–8 HRT after inoculation, an important part of the nitrogen disappeared during the aerobic phase in comparison with that of control cycles: 33% for the anoxic inoculum and 42% for the aerobic one (Table 2). However, after a much longer period, the nitrogen distribution was similar to that in the control cycles. It seemed that the bioaugmentation with *M. aerodenitrificans* cells, adapted or not, enhanced the performances in terms of aerobic nitrate removal without any perturbation of the phosphate removal yield. However, this aerobic denitrifying activity decreased within 4 days, which corresponded to a decrease in the number of cells hybridized with the two specific probes. It is however difficult to choose between the two conditions of inoculation. As it is easier to obtain large culture volume under aerobic conditions than anoxic one, inoculum was realized under oxic conditions for further experiments.

The second experiment consisted in increasing the amount of cells added to the reactor, with proportion of *M. aerodenitrificans* amongst the total biomass of 3.2, 10.3 and 15%. The lower supply led to a maximum loss of nitrogen after 6 HRT. The supply at 10.3% gave the best compromised results in terms of nitrogen loss during the aerobic phase as well as in terms of longer presence time. Indeed, after 20 HRT (10 days), the presence of the pure strain and an aerobic denitrifying activity were always detected. To the opposite, the 15% supply gave worst results with a loss of nitrogen quite close to that obtained on the acetate test at 8 HRT (Tables 1 and 2). Moreover, FISH revealed that this massive inoculation induced a rapid decline in the proportion of *M. aerodenitrificans* amongst the free-living bacteria and the flocs within 1 day. Three days later, isolated cells were still present, but after 4 days more they were in such low numbers that they could no longer be accurately quantified.

The third experiment compared the previous single supplies to a small but daily supply, equivalent to 1.6% amongst the total biomass the first day and to 1% during 29 more days. Table 2 shows that a nitrogen loss was observed while the pure strain was added, but 2 days after the stop of the supplies, no more aerobic denitrifying activity was noticed. The percentage of disappeared nitrate varied between 15 and 26%, which was quite inferior to the maximum loss observed with the 3.2 or 10% single *M. aerodenitrificans* supply. Activity and presence of *M. aerodenitrificans* were again correlated: the quantity of hybridized *M. aerodenitrificans* stabilized while it was supplied, and then declined. However, fifteen days after the stop of inoculation, cells remained hybridized with the two *M. aerodenitrificans* specific probes.

Whatever the manner and the amount, it appeared that the majority of the cells were eliminated of the reactor during the drawing step. Moreover, the *M. aerodenitrificans* addition was well correlated to an increase in the protozoa population and microscopic examination revealed that their digestive vacuoles were full of the added bacteria emitting a positive signal with the probes. However,

Table 3. Nitrogen balance during the test of inoculation of *M. aerodenitrificans* embedded in alginate beads

	HRT (day) after *M. aerodenitrificans* inoculation				
	2	8	12	30	40
%N-oxides	4	2	1.8	−22	3.4
%N-NH_4^+	47.5	41.1	47.9	33.2	28.5
%N-assimilation	7.5	5.8	8.7	10.5	9.9
$\%\Delta N_{anoxic}$	0	2.5	0	0	0
$\%\Delta N_{aerobic}$	40	48.6	41.6	78.5	58.2

during their presence in the reactor, the aerobic denitrifiers exhibited an activity which allowed to release an effluent with a concentration of N-oxides varying between 10.2 and 13.8 mg/l, a concomitant phosphate concentration varying between 0 and 2 mg/l and no ammonia.

The last experiment consisted in the use of alginate beads as a vector of introduction of *M. aerodenitrificans* in the complex ecosystem. The amount of embedded cells added was equivalent to 22% of the total biomass. Results are summarized in the Table 3 and Fig. 2. Phosphate removal performances varied between 40 and 50%, but they were never affected by the *M. aerodenitrificans* bioaugmentation. Moreover, acetate was not completely consumed during the anaerobic phase, leading to a residual concentration (30 mg/l of C-acetate) at the beginning of the aerobic period. Thus, no acetate was added for aerobic denitrification. Moreover, nitrification was not total. Indeed, only 58–72% of ammonium was consumed implying a residual ammonium concentration in the effluent around 20 mg/l. However, it is interesting to note that a very small quantity of N-NO_x were produced during the aerobic phase compared to the consumption of ammonium. This lead to a N-NO_x mean value in the effluent around 5 mg/l with a maximum at 12 mg/l (Fig. 2). Consequently, high percentages of aerobic denitrification were calculated, between 40 and 80%. The higher one (78.5%) was measured 15 days after addition of the pure strain. During this cycle, the N-NO_x produced by nitrification were immediately consumed by the aerobic denitrifier and the residual N-NO_x concentration present at the beginning of the anaerobic phase was also consumed. These facts explained the negative value of the %N-oxides. The aerobic denitrifying performances were observed up to the 20th day following inoculation and was well correlated to the FISH results. Indeed, these later showed that *M. aerodenitrificans* was always present in the sludge whereas the alginate beads broke down 9 days after their introduction. Twenty days after the bioaugmentation, the relative number of *M. aerodenitrificans* among the total bacterial population was estimated to be 5%. Moreover, it seemed that *M. aerodenitrificans* had colonized the phosphate removal sludge.

DISCUSSION

Objectives of this study were twofold: to enhance performances of a nutrient removal SBR and to follow the fate in terms of maintenance and activity of a pure aerobic denitrifier, *M. aerodenitrificans* introduced in a complex ecosystem. First experiments were realized in order to be sure that the introduced cells had no direct inhibitory effect on the P-accumulating activated sludge and to know the real impact of acetate addition at the beginning of the aerobic phase, this addition being necessary for the heterotrophic denitrifier to reduce aerobically the N-oxides. The studies of bioaugmentation were always compared to these control tests. A continuous supply of acetate during the first hour of the aerobic phases of the SBR cycle had no effect

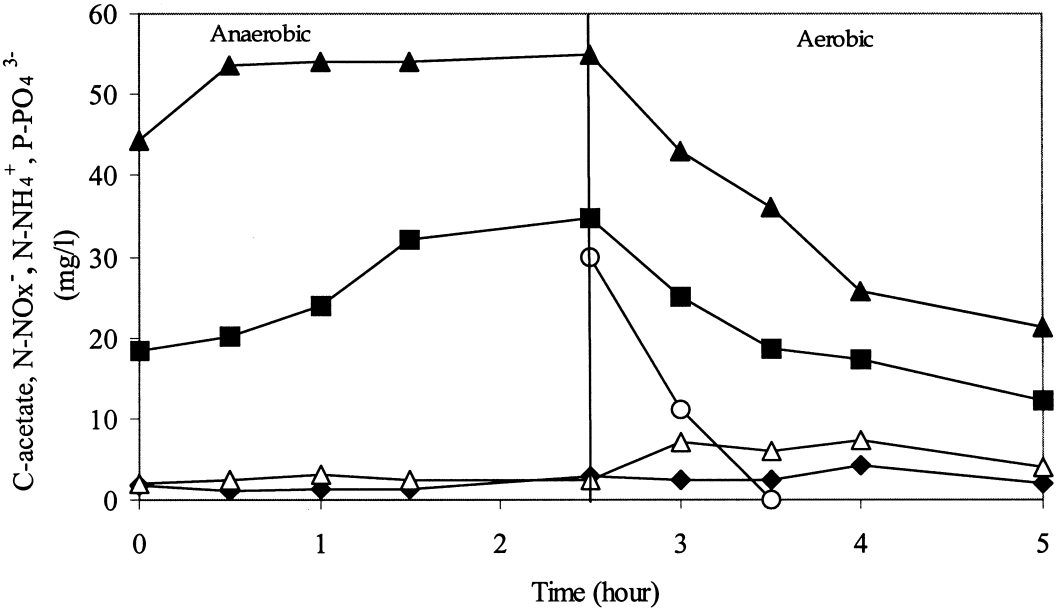

Fig. 2. Phosphate, N-oxides, ammonium and C-acetate profiles of a cycle 6 days after the inoculation of *M. aerodenitrificans* embedded cells. Δ $N\text{-}NO_3^-$, ◆ $N\text{-}NO_2^-$, ■ $P\text{-}PO_4^{3-}$, ▲ $N\text{-}NH_4^+$, ○ C-acetate (mg/l).

on phosphate removal performances but increased the growth of an heterotrophic biomass at the expense of the nitrifying activity. Moreover, an aerobic denitrifying activity was measured whereas no *M. aerodenitrificans* was added to the system. On one hand, this aerobic activity could be explained by an under-estimation of the N assimilation calculated through the growth yield. This later parameter was difficult to evaluate because periodic sludge withdrawals/sampling have to be realized in order to maintain a defined age-sludge (52 days), which is a key parameter of a good P-accumulating sludge quality in terms of settling and activity (Rodrigo *et al.*, 1996). On the other hand, it could be the expression of indigenous aerobic denitrifying bacteria. FISH results are in accordance with this suggestion. Indeed, Mae-1414 and Mae-0829 FISH showed that *M. aerodenitrificans* was initially present in the phosphate removal sludges. Furthermore, a recent study showed that aerobic denitrifiers are usually present in ecosystems subjected to alternating oxic-anoxic phases (Patureau *et al.*, 2000b). It is thus not surprising to observe an aerobic denitrifying activity in this case. Because the strain was still present in the sludge and because its residual activity was too low, the following experiments consisted in enhancing its indigenous activity by introduction of an activated *M. aerodenitrificans* biomass.

Introduction of *M. aerodenitrificans* was always followed by an increase of the aerobic denitrifying capability of the complex ecosystem. In terms of performances, the nitrogen amount in the effluent (10–14 mg/l) linked close to the European quality standard. Higher aerobic denitrifying rates could be obtained by working with higher nitrate concentrations. Indeed, a recent study showed that level of oxygen, nitrate and carbon concentrations are key factors for observing aerobic denitrification (Patureau *et al.*, 2000a). These operational factors were however not tested in the case of phosphorus removal systems. However, the aerobic denitrifying activity well correlated to the presence of *M. aerodenitrificans* maintained 8 days. The relative number of introduced *M. aerodenitrificans* decreased to finally reach the value calculated on the control system. On one hand, the feasibility of combining nitrification and denitrification in a single aerated unit was still demonstrated, on the other hand, the effectiveness of bioaugmentation fell still under debate. Some full-scale experiments with bioaugmentation have been reported as being successful (Stephenson and Stephenson, 1992; Rittmann and Whiteman, 1994). However, in these cases, the target activity maintained because bioaugmentation was daily repeated. The goal of our study was completely different: through one or two additions and by applying specific culture conditions, we would like to introduce the strain to the received ecosystem and by this way to enhance the community already present. The failure of our experiment might have several origins. The grazing by protozoa was a well known factor of introduced cells disappearance (Eberl *et al.*, 1997; Bouchez *et al.*, 2000a). A visual estimation of the increased number of protozoa after *M. aerodenitrificans* bioaugmentation leads to a similar conclusion. The competition for the carbon and ammonium between *M. aerodenitrificans*, the indigenous heterotrophs and the nitrifiers may be another failure reason. Moreover, the non settling capability of *M. aerodenitrificans* involved a massive elimination of the introduced microorganisms during the withdrawal periods. Problems of adaptation of the inoculated microorganisms to the new medium or the new partners may not be a failure reason because the strain has still its niche in the sludge and because a good adequation was found between the added strain, the received ecosystem and the conditions applied.

Bioaugmentation for soil remediation is subjected to the same kind of difficulties: diversity and complexity of the microorganisms used and of the received ecosystem, environmental heterogeneity (pH, organic matter content, presence of co-substrates, humidity), bioavailability substrate-inoculated bacteria (insolubility, low level of concentration, biomass mobility and loss). In order to improve the efficacy of bioaugmentation in terms of favoring the contact between the microorganisms and their target substrate, some authors proposed to fix the activated biomass on Gellan gum beads (Norton and Lacroix, 1990) to remove toxic organic compounds from an aquifer. Van Veen *et al.* (1997) proposed the use of carrier materials like peat, clay, calcium alginate, agarose and k-carrageenan to improve the establishment and efficacy of microbial inoculants in soils. In the same way, alginate beads were used as a physically protective niche to favor the introduction of *M. aerodenitrificans* into the activated sludge, to protect it against protozoa grazing and to evade the dilution effect of the withdrawals. This inoculation strategy reached the two objectives: (1) survival: inclusion of *M. aerodenitrificans* into the floc structure with a longer shelf life compared to that obtained with other inoculation experiments carried out with *M. aerodenitrificans* in a complex nitrifying ecosystem (Bouchez *et al.*, 2000a,b) and simultaneously (2) activity: observation of the highest aerobic denitrifying performances that allows the proposition of a nutrient removal process in two steps less complex than those previously proposed processes (Cooper *et al.*, 1994).

CONCLUSIONS

This paper proposed a more integrated configuration for nitrogen and phosphorus removal through the introduction of an aerobic denitrifier in a com-

plex phosphorus/nitrifying ecosystem. It provides a more complete overview on the phenomenon of aerobic denitrification and a more complete understanding on the mechanism of bioaugmentation of a pure strain in a complex ecosystem.

Aerobic denitrification was already combined to nitrification in single aerobic reactors like continuous stirred reactor or sequencing batch reactor, however this was the first time that it was combined to a more complex ecosystem and applied to urban wastewater like effluent. Several conclusions can be drawn from this work:

1. Aerobic denitrification is effective in the phosphate/nitrifying ecosystem and allowed to obtain an effluent with a standard quality: nitrate disappearance during the aerobic phase may only be due to respiration. No nitrite accumulated in the system. It would be interesting however to determine if other denitrifying intermediates accumulated, like nitrous oxide.
2. Enhancement of the aerobic denitrifying activity can be obtained by modifying the ratio nitrate onto oxygen. Indeed, it was demonstrated that the aerobic denitrifying percentage of *M. aerodenitrificans* increased with decreased dissolved oxygen concentrations. This latter can be optimized in order to not inhibit nitrification and to improve aerobic denitrification.
3. The aerobic denitrifying activity was linked to the presence of high number of *M. aerodenitrificans* cells: disappearance of *M. aerodenitrificans* or low cells number implied no activity. The disappearance of the cells was essentially due the type of the reactor used and the obligate withdrawal phase. Strategies of bioaugmentation had to be well defined in order to definitively incorporate the denitrifying cells into the indigenous biomass and to protect it again protozoa grazing too.
4. The strategy which gave the best compromise between phosphorus/nitrogen removal performances and strain maintenance consisted of continuous carbon source addition during the first hour of the aerobic phase and of addition of *M. aerodenitrificans* embedded in alginate beads. Alginate beads can be then proposed as a privileged vector of bioaugmentation for biological removal processes.

This research contributes then to enlarge the view on bioaugmentation that could be considered as a successful way to improve performances of wastewater treatment processes.

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