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Effect of dissolved oxygen and carbon–nitrogen loads on denitrification by an aerobic consortium

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Abstract Four samples of natural ecosystems and one sample from an activated sludge treatment plant were mixed together and progressively adapted to alternating aerobic/anoxic phases in the presence of nitrate in order to enrich the microflora in aerobic denitrifiers. Aerobic denitrifying performances of this mixed ecosystem at various dissolved oxygen concentrations and various carbon–nitrogen loads were evaluated and compared to those obtained with the aerobic denitrifier *Microvirgula aerodenitrificans*. The consortium and the pure strain exhibited an aerobic denitrifying activity at air saturation conditions (7 mg dissolved oxygen l^{-1}), i.e. there was co-respiration of the two electron acceptors with significant specific nitrate reduction rates. Dissolved oxygen concentrations had no influence on denitrifying performances above a defined threshold: 0.35 mg l^{-1} for the consortium and 4.5 mg l^{-1} for *M. aerodenitrificans* respectively. Under these thresholds, decreasing the dissolved oxygen concentrations enhanced the denitrifying activity of each culture. The higher the carbon and nitrogen loads, the higher the performance of the aerobic denitrifying ecosystem. However, for *M. aerodenitrificans*, the nitrate reduction percentage was affected more by variations in nitrogen load than in carbon load.

Introduction

Nitrogen level evaluation in natural environments such as soils, or engineered ecosystems such as wastewater treatment plants, is usually complicated by the interacting web of reactions involved. However, the different reactions, the groups of microorganisms and the physicochemical conditions of the nitrogen cycle steps are well known and described. Until now, to establish a nitrogen balance in a wastewater treatment plant, nitrification and denitrification are considered independently and lots of calculations are made without considering interactions between these two reactions. However, for the past ten years, many studies have reported the existence of atypical behavior for nitrogen-fixing, nitrifying, ammonifying and denitrifying bacteria. This unusual and unexpected behavior could explain some disturbances and nitrogen imbalances observed in wastewater treatment plants and in soils. For example, some microorganisms are capable of heterotrophic nitrification (Kuenen and Robertson 1987). It was observed that many common denitrifying bacteria present in soils or in wastewater treatment plants are also heterotrophic nitrifiers (Castignetti and Hollocher 1984; Robertson et al. 1989; Van Niel et al. 1992). Other examples of new metabolic pathways are the Anammox reaction (Jetten et al. 1999), the anaerobic reduction of nitrogen oxides into nitrogen gas by nitrifiers (Remde and Conrad 1990; Abeliovich and Vonshak 1992; Schmidt and Bock 1997) and the aerobic reduction of nitrogen oxides by denitrifiers (Robertson and Kuenen 1990; Bonin and Gilewicz 1991; Patureau et al. 1994). These new reactions, in addition to explaining nitrogen imbalances, allow the design of new nitrogen-removal systems. 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.

For more than six years, our laboratory has been working on aerobic denitrification, especially on a Gram-negative strain named *Microvirgula aerodenitrificans* isolated from an upflow anoxic/aerobic filter (Patureau et al. 1998). Despite isolation of other aerobic denitrifiers (Carter et al. 1995; Frette et al. 1997; Lukow and Diekmann 1997; Patureau et al. 2000), implying a widespread occurrence of this kind of microorganisms in soils, sediments and wastewater treatment plants, it remains unclear how the two pathways (oxygen and nitrate

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respiration) can work concomitantly as was demonstrated through kinetic experiments (Patureau et al. 1996a). In order to shed light on some physiological points of aerobic denitrification, a consortium was obtained from mixed natural and engineered environments by applying alternating aerobic/anoxic phases in the presence of nitrate. This study focuses on kinetic aspects and performances of the consortium. The influence of dissolved oxygen (DO) concentration and of carbon–nitrogen loads on aerobic nitrate reduction rate was studied in continuous culture. These results were compared both to the data obtained with *M. aerodenitrificans* under the same culture conditions and to data from other nitrogen-removal systems, in order to assess the importance of aerobic denitrification in the nitrogen cycle.

Materials and methods

Sample sites

Sample sites and sampling conditions were the same as described in Patureau et al. (2000). Five samples were obtained from ecosystems where aeration conditions are assumed to fluctuate in space or in time. Solid samples were collected from two soils. Liquid samples were collected from a canal and a pond. A further sample was collected from the aerobic basin of the municipal wastewater treatment plant.

Enrichment in denitrifiers

Samples were first independently enriched in denitrifiers under anoxic conditions (Patureau et al. 2000). Concentrated cell suspensions of each of the five enriched cultures were mixed together in order to optimize the number of denitrifying bacteria. The mixture was used to inoculate a 2-l Biolafitte reactor filled with 1.5 l of the growth medium described in Patureau et al. (1996b). The medium was supplemented with 250 mg nitrate l^{-1} of and 500 mg carbon source l^{-1} (equal concentrations of acetate, propionate and ethanol). A dilution rate of 0.033 h^{-1} was used throughout the study, corresponding to a hydraulic retention time (HRT) of 30 h. The medium was stirred at a constant rate of 700 rpm. Temperature and pH were regulated at 35 °C and 7, respectively. The population was progressively adapted to aerobic denitrifying conditions by increasing the aerobic period time from 2 h $week^{-1}$ to 7 days in every 14 days. This adapted mixture was then used to evaluate the aerobic performance of the consortium.

Test conditions on the aerobic consortium

Variable DO concentrations were applied by bubbling the medium with a known flow rate (200 ml min^{-1}) of a mixture of air and argon. The DO concentrations were measured by a polarographic-type electrode connected to an Ingold transmitter (type 170, % air). The sterilized feeding medium was continuously bubbled with argon to avoid nitrogen contamination from air, which could cause errors in calculating the nitrogen balance. At a constant DO concentration, different carbon–nitrogen loads were tested by modifying the carbon and nitrate concentrations in the feeding medium. Each test was applied during 7 HRT in order to be sure to observe stable phenomenon.

M. aerodenitrificans culture conditions

The continuous culture of *M. aerodenitrificans* (LMG 18919) was carried out in the same kind of 2-l reactor, filled with 1.5 l of the growth medium (Patureau et al. 1996b) with variable concentrations of nitrate and carbon source (acetate). The medium was stirred at a constant rate of 700 rpm. Temperature and pH were regulated at 35 °C and 7, respectively. A dilution rate of 0.042 h^{-1} was used throughout the study, corresponding to a HRT of 24 h. The reactor was inoculated with an overnight aerobic pre-culture of *M. aerodenitrificans* adapted to nitrate. The different aeration conditions were obtained by the same air/argon mixture as mentioned above. The chemostat culture was first submitted to aerobic conditions (7 HRT), then to anoxic (7 HRT) and finally to aerobic (7 HRT). Variable carbon–nitrogen loads were tested at 3.5 mg DO l^{-1} during 7 HRT for each concentration. Batch experiments were also carried out in the same kind of reactor under strictly anoxic and aerobic (without nitrate) conditions and with the same medium (250 mg nitrate l^{-1}, 500 mg acetate l^{-1}) in order to determine the anoxic and aerobic yields (mg particulate chemical oxygen demand [COD]/mg COD consumed) and the COD/volatile suspended solids (VSS) ratio specific to *M. aerodenitrificans*.

For the two cultures, calculations are made as follows. The percentage of nitrate reduction is calculated from the formula:

$$([\text{N-NO}_x]i - [\text{N-NO}_x]e)/[\text{N-NO}_x]i \times 100$$

where i is influent, e is effluent and nitrogen oxides NO_x is nitrate and nitrite.

By knowing the quantity of nitrogen oxides consumed, the COD consumed for the reduction of nitrogen oxides (COD_{cons/NO_x}) was calculated using the stoechiometric ratio COD/N = 2.86 (at steady state N was nitrate):

$$COD_{cons/NO_x} = 2.86 \times ([\text{N-NO}_x]i - [\text{N-NO}_x]e)$$

Copp and Dold (1998) have calculated two different biomass yield for an activated sludge: an aerobic yield of 0.645 mg particulate COD mg^{-1} consumed COD and an anoxic yield of 0.402 mg particulate COD mg^{-1} consumed COD. We hypothesize that the two ways of respiration act independently. These values then allow calculation of the repartition of COD through biomass production ($COD_{cons/particulate/total} = COD_{cons/particulate[nitrate]} + COD_{cons/particulate[oxygen]}$) and through electron acceptor consumption (COD_{cons/NO_x} and $COD_{cons/oxygen}$) using the following equations:

$$COD_{cons/particulate(nitrate)+cons/NO_x} = COD_{cons/NO_x}/(1 - 0.402)$$

$$COD_{cons/particulate(oxygen)+cons/oxygen} = COD_{total\ cons} - COD_{cons/particulate(nitrate)+cons/NO_x}$$

$$COD_{cons/oxygen} = (1 - 0.645) \times COD_{cons/particulate(oxygen)+cons/oxygen}$$

$$COD_{cons/particulate/total} = (0.645 \times COD_{cons/particulate[oxygen]+cons/oxygen}) + (0.402 \times COD_{cons/particulate[nitrate]+cons/NO_x})$$

For *M. aerodenitrificans*, an anoxic yield of 0.467 mg particulate COD mg^{-1} consumed COD, an aerobic yield of 0.532 mg particulate COD mg^{-1} consumed COD and a ratio of 1.05 mg COD mg^{-1} VSS were evaluated through the batch experiments. These different calculations allowed the evaluation of the carbon fluxes toward nitrogen compounds and/or oxygen, which are thought to be consumed simultaneously. Consumption of ammonia was also measured and compared to nitrogen assimilation evaluation through biomass concentration measurement considering that 9–12% of biomass is made of nitrogen.

Analytical measurements

Samples were centrifuged at $17{,}500 \times g$ (4 °C, 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). Carbon sources were analyzed by gas chromatography. VSS were calculated after filtration of a 10-ml sample according to standard methods.

Results

Influence of DO concentrations on the aerobic denitrifying performances of the consortium

Using chemostat culture at a fixed dilution rate with stable loads of nitrogen (165 g nitrate m^{-3} day^{-1}) and carbon (363 g carbon m^{-3} day^{-1}), a series of steady states was established at different mixtures of air and argon. Each aerating condition corresponded with a constant and non-limiting flow rate of an argon–air mixture. The DO concentrations were always close to those expected from the argon–air mixture. This implies that aeration rates were so high that the oxygen consumption had only a negligible effect on the DO. The carbon source was entirely consumed. The consumption of $N\text{-}NH_4^+$ (which was not a limiting factor) was well correlated to the nitrogen concentration in biomass. Nitrate consumption was observed whatever the aerating conditions used. The $N\text{-}NO_2^-$ concentration in the effluent varied over 0–3.3 mg l^{-1}. The highest nitrite concentration was observed at the beginning of each air mixture modification. At steady state, the nitrite concentration was zero. The disappearance of nitrate was then well correlated to denitrifying activity with small transitory accumulations of nitrite. Table 1 summarizes the different calculations made at each steady state. At 1.7–6.3 mg DO l^{-1}, the aerobic denitrifying percentage leveled off around 31%. When decreasing DO concentrations were applied, the nitrate reduction rate increased gently from 31% to 36%. However, under 0.35 mg l^{-1}, the nitrate reduction rate increased sharply from 36% to 86%. As soon as higher DO concentrations were applied, the denitrifying percentage fell (but more gradually) to reach 37% at 4 mg DO l^{-1}. It seems that the aerobic denitrifying performances of the consortium were completely independent of DO concentrations above a defined, low threshold and corresponded approximately to 49.5 g nitrate m^{-3} day^{-1} (30%). However, after anoxic acclimatization of the biomass, it seems that aerobic denitrifying performances were enhanced. Indeed, for the same DO value (0.33 mg l^{-1}), the nitrate reduction percentages were different: 36% if changed from aerobic to anoxic and 65% if changed from anoxic to aerobic condition, whereas a steady state had been achieved. The experience was repeated with observations giving the same answer.

The constant nitrate reduction percentage corresponded with a constant part of COD being oxidized by nitrate (13–15%). Biomass production was linked to the concomitant consumption of electron acceptors, here nitrate and oxygen, and consumption of electron donors. Because of a constant COD load and because of a constant COD flux to nitrate, it was observed that a constant part of COD was used to reduce oxygen and a constant part of COD was used to produce biomass. The part of COD used to produce particulate COD biomass (58%) logically comprised both the aerobic particulate (64.5%) and the anoxic (40.2%) yields. The biomass yield calculated here was the result of aerobic and anoxic growth, but with a much more pronounced tendency to oxygen. This showed that, despite the consumption of nitrate, oxygen remained the preferred electron acceptor thus favoring a higher biomass production.

Under the threshold of 0.35 mg DO l^{-1}, which corresponded to higher denitrifying performances, the COD repartition was modified with a smaller flux to oxygen, a higher flux to nitrate and a lower flux to biomass (49%). Biomass production was thus the result of a much greater diversion of electrons to nitrate than to oxygen, although the latter however was still consumed. As soon as the DO increased, the carbon flux was diverted to oxygen and leveled off at 29%. At the end of the experiment, the COD repartition was the same as at the beginning. This corresponded to a typical aerobic profile: 30, 10 and 60% respectively for $COD_{cons/oxygen}$, COD_{cons/NO_x} and $COD_{cons/particulate/total}$.

The behavior of *M. aerodenitrificans* under the same culture and aeration conditions was exactly the same,

Table 1 Influence of dissolved oxygen (DO) concentrations on denitrifying activity (expressed in % of nitrate reduction) and on chemical oxygen demand (COD) repartition (energy or biomass pathway) for the consortium. Aeration conditions, tested in the order shown, were applied for 7 hydraulic retention times (HRT), with the exception of the first partial aerobic period at 1.7 mg l^{-1}, which was applied for 1 month. COD fluxes are expressed in % because the COD load was constant throughout the experiment. Values are means calculated over 3 days after steady state was achieved (standard deviation: ±2%). DO was measured with a polarographic electrode

Aeration conditions, DO (mg l^{-1})	% Nitrate reduction	$COD_{cons/oxygen}$ (%)	COD_{cons/NO_x} (%)	$COD_{cons/biomass\ total}$ (%)
1.7	31	27	13	60
4.0	29	27	14	59
6.3	33	26	15	59
4.1	35	27	15	58.2
0.35	36	25	16	58
0.1	56	21	25	54
0	86	13	38	49
0.33	65	19	27	54
1.2	64	25	18	57
4.09	37	30	10	60

but with changed values (Table 2). The nitrate reduction percentage remained constant for high DO concentrations but the value was lower than that calculated with the consortium. It comprised 5.3–6.15%, compared to 31% for the consortium. However, from 4.5 mg l^{-1}, the percentage increased gently to 8.4% at 2.2 mg l^{-1}; and it tripled to 14.8% at 1 mg l^{-1}, to reach 100% at 0 mg l^{-1}. After the anoxic switch, it maintained high values until 1.6 mg l^{-1}, when it fell to 1%. The evolution of the percentage of $COD_{cons/NOx}$ followed exactly the same pattern as the nitrate reduction rate: it was low and constant (1.5%), then it progressively increased to reach a maximum under anoxic conditions (53%) and decreased as soon as oxygen was supplied, but with less extent than after 1.6 mg l^{-1} where it reached 1%. Consequently, the COD flux to oxygen and the COD flux to biomass fluctuated respectively between 45% and 0% and between 47% and 53%.

Influence of carbon–nitrogen loads on the aerobic denitrifying activity of the consortium

Experiments were carried out at a fixed argon–air mixture (50–50) and a constant aeration flow rate (200 ml h^{-1}) corresponding to a measured DO concentration of 3.5 mg l^{-1}. Series of steady states were established at different COD loads with constant nitrogen load and at different nitrogen loads with constant COD load. The results are summarized in Table 3. At constant nitrogen load (condition [a]), it appeared that the nitrate reduction percentage and the nitrate-disappeared rate evolved in parallel to the COD load. Indeed, a half decrease in COD load implied a sharp denitrifying percentage fall from 36% to 2%. In contrast, increasing the COD load increased the denitrifying percentage to reach 76% at high COD load. For higher COD load (5.3 kg m^{-3} day-1), 100% of denitrification occurred and oxygen became the limiting factor with transfer and consumption of the overall quantity of oxygen supply, implying a DO concentration of 0 mg l^{-1}. COD load fluctuations were tested too at lower nitrogen load (36.5 g nitrate m^{-3} day^{-1}): it appeared that doubling or tripling the COD load had no effect on the nitrate reduction percentage, which remained at 0%. This implied that nitrogen concentration level is an important factor to observe or not aerobic denitrifying activities. Moreover, at a constant carbon load (1.1 kg m^{-3} day^{-1}), nitrogen load fluctuations had an influence on aerobic denitrifying performances: increasing nitrogen loads implied higher performances in terms of percent of nitrate reduced (10–56%) as well as in terms of quantity reduced.

The higher the COD load, the higher the fluxes of COD used to reduce oxygen and nitrate. There was a good correlation between the two electron acceptor uptake rates and the electron donor consumption rate (data not shown). However, these observations were only verified when the system was subjected to increased carbon loads. Indeed, decreased COD loads had higher impact on aerobic denitrifying activity than on oxygen respiration. For evidence, at similar COD and nitrogen loads (approximately 4 kg m^{-3} day^{-1} and 165 g nitrate m^{-3} day^{-1}), a 134 g nitrate m^{-3} day^{-1} uptake rate was calculated during a period of increasing carbon loads, against 41 g nitrate m^{-3} day^{-1} during a period of decreasing carbon loads.

At constant COD load, increasing nitrogen loads implied higher electron diversion from carbon to nitrate varying over 41–399 g COD_{cons/NO_x} m^{-3} day^{-1}, accompanied by a lower diversion to oxygen and a smaller growth yield.

In comparison, the behavior of *M. aerodenitrificans* towards carbon–nitrogen loads variations was completely different (Table 4). It seemed that the denitrifying activity was less affected by COD load fluctuations than by nitrogen load fluctuations. Indeed, the nitrate reduction percentage increased gently from 26% to 33%, which corresponded to a slightly higher nitrate uptake rate (69 g nitrate m^{-3} day^{-1}), whereas the COD load decreased by half. In contrast, a smooth decrease in the COD load and a half decrease in the nitrogen load corresponded with a sharp decrease in the denitrifying rate. It is important to underline that the nitrate reduction percentage varied less (between 26% and 35%) than the denitrifying rate.

Table 2 Influence of DO concentration on denitrifying activity and COD repartition (energy or biomass pathway) for *M. aerodenitrificans*. Aeration conditions, tested in th order shown, were applied for 7 HRT. The COD fluxes are expressed in % because the COD load was kept the same throughout the experiment. Values are means calculated over 3 days after steady state was achieved (standard deviation: ±2%). DO was measured with a polarographic electrode

Aeration conditions, DO (mg l^{-1})	% Nitrate reduction	$COD_{cons/oxygen}$ (%)	COD_{cons/NO_x} (%)	$COD_{cons/biomass\ total}$ (%)
6.7	6.15	45.0	2	53.0
4.5	5.3	46.0	1	53.0
2.2	8.4	44.0	3	53.0
1.0	14.8	41.0	7	52.0
0.2	85.8	22.0	28	50.0
0	100.0	0.25	53	46.7
1.6	95.5	25.0	25	50.0
3.2	0.8	46.6	0.2	53.2
5.5	1.0	46.6	0.2	53.2

Table 3 Influence of COD and nitrogen load on denitrifying activity (expressed in nitrate reduction percentage and in nitrate-disappeared rate) and COD repartition (energy or biomass pathway) for the consortium. All conditions were applied for 7 HRT. The sum of COD repartition values gives the daily $COD_{cons/total}$, equivalent to the COD load. Values are means calculated over 3 days after steady state was achieved (standard deviation: ±2%)

	Denitrifying activity		$COD_{cons/oxygen}$ (g m^{-3} day^{-1})	COD_{cons/NO_x} (g m^{-3} day^{-1})	$COD_{cons/biomass\ total}$ (g m^{-3} day^{-1})
	%	Rate (g m^{-3} day^{-1})			
COD load (g m^{-3} day^{-1})					
2,042[a]	36	61	625	174	1,243
1,037[a]	2	3	362	9	666
2,436[a]	27	46	775	131	1,530
207[b]	0	0	73	0	134
442[b]	0	0	157	0	285
552[b]	0	0	196	0	356
2,446[a]	34	55	772	157	1,517
4,058[a]	76	134	1,210	382	2,466
5,367[a,d]	100	170	1,584	487	3,203
4,499[a]	40	63	1,490	179	2,830
3,728[a]	26	41	1,252	117	2,359
N load (g m^{-3} day^{-1})					
145[c]	10	14.5	375	41	742
249[c]	56	139	199	399	560

[a] N load: 156–176 g m^{-3} day^{-1}
[b] N load: 32–40 g m^{-3} day^{-1}
[c] COD load: 1,100 g m^{-3} day^{-1}
[d] A small quantity of COD was not consumed and DO was 0 mg l^{-1}

The increase of nitrate uptake rate was always accompanied by an increase in the COD flux to nitrate. However, the other COD fluxes were much more affected by the COD load fluctuations than nitrogen load fluctuations. Indeed, the drastic decrease in the COD load was accompanied by high falls in the $COD_{cons/oxygen}$ and $COD_{cons/biomass\ total}$ and little increase in the COD_{cons/NO_x}.

Discussion

It was currently assumed that denitrification only occurred under anoxic conditions. Applying increased DO concentrations to anoxic denitrifying cells was shown to be accompanied by a decrease in denitrifying rates and accumulation of nitrous oxide, nitric oxide, nitrite and then nitrate (Hochstein et al. 1984). However, for more than ten years, many experiments have shown that activity and synthesis of denitrifying enzymes can occur under oxic conditions (Robertson et al. 1989; Patureau et al. 1994; Carter et al. 1995; Frette et al. 1997; Lukow and Diekmann 1997). In this paper, physiological aspects, i.e. behavior towards a variation in the concentration of electron acceptors and donors, were tested with an ecosystem adapted to alternating aerobic/anoxic conditions. The first assumption was that aerobic denitrification is the rule rather than the exception (Lloyd et al. 1987). Indeed, as it was soon demonstrated (refs), after six months of adaptation, whatever high DO concentration was applied, nitrate was always reduced simultaneously to oxygen, without the production of intermediates (Patureau 1995; Patureau et al. 1996b). This nitrate disappearance could be due to assimilatory reduction of nitrate into ammonium. However, this pathway was assumed to be inhibited by the ammonium that was always present in our experiment. The dissimilatory pathway (reduction of nitrate into ammonium) usually occurs under reducing conditions which did not correspond to our experiments. So, nitrate reduction could only be due to a respiratory process occurring simultaneously to oxygen reduction. Consequently,

Table 4 Influence of COD and nitrogen load on denitrifying activity (expressed in nitrate reduction percentage and in nitrate-disappeared rate) and COD repartition (energy or biomass pathway) for *M. aerodenitrificans*. Conditions were applied for 7 HRT. The sum of COD repartition values gives the daily $COD_{cons/total}$, equivalent to the COD load. Values are means calculated over 3 days after steady state was achieved (standard deviation: ±2%)

	Denitrifying activity		$COD_{cons/oxygen}$ (g m^{-3} day^{-1})	$COD_{cons/NOx}$ (g m^{-3} day^{-1})	$COD_{cons/biomass\ total}$ (g m^{-3} day^{-1})
	%	Rate (g m^{-3} day^{-1})			
COD load (g m^{-3} day^{-1})					
3,472[a]	26	55	1,485	158	1,829
1,653[a]	33	69	598	196	859
N load (g m^{-3} day^{-1})					
185[b]	35	65	1,174	185	1,500
96[b]	29	28	1,030	80	1,241

[a] N load: 208–212 g m^{-3} day^{-1}
[b] COD load: 2,351–2,859 g m^{-3} day^{-1}

carbon is consumed to reduce both nitrate and oxygen, and to produce biomass. In terms of energy conservation, the co-uptake of oxygen and nitrate implies a growth yield halfway between the aerobic and anoxic one. For the consortium and *M. aerodenitrificans*, the extent of energy conservation during oxygen–nitrate respiration was respectively about 90% and 98% of that during aerobic respiration. It was currently stated that this extent during nitrate respiration was about 70% of that during aerobic respiration (Stouthamer 1991). The higher growth yields corroborates again the idea of a two-way electron flux.

In terms of nitrogen-removal performances, the denitrifying rates were 0.122 and 0.066 kg nitrate m^{-3} day^{-1} respectively for the consortium and *M. aerodenitrificans*. These rates were calculated for low nitrogen loads (0.16 kg nitrate m^{-3} day^{-1}). However, according to the data obtained with increasing nitrogen loads on the consortium and on *M. aerodenitrificans*, it seems that better performances could be obtained at higher nitrogen loads. *M. aerodenitrificans* at a nitrogen load of 1.4 kg nitrate m^{-3} day^{-1} had a nitrate-removal rate of 0.35 kg nitrate m^{-3} day^{-1}; i.e. a specific activity of 1.75 kg nitrate kg^{-1} dry weight day^{-1}. In comparison (Table 5), anoxic denitrification rates varied over 0.012–0.32 kg nitrate kg^{-1} dry weight day^{-1} with volumetric performances varying over 0.8–6 kg nitrate kg^{-1} dry weight day^{-1} (Bode et al. 1987; Martins dos Santos et al. 1998). The largely lower volumetric performances of aerobic denitrification compared to those of anoxic denitrification were caused by the low sludge/biomass concentration (about 1 g l^{-1}) and were not due to a low level of microorganism activity. The biomass concentration was intentionally maintained at this low value in order to be sure of having no anoxic zones in the reactor. Thus, the specific aerobic denitrifying activities were always: (1) higher to those obtained with nitrifiers, (2) largely superior to those obtained with anaerobic nitrifiers and (3) comparable to those obtained with anoxic denitrifiers and with the Anammox biomass (Table 5). Van Loosdrecht and Jetten (1998) stated that heterotrophic nitrification/aerobic denitrification can be considered negligible in wastewater treatment processes. Their statement was based on reported *T. pantotropha* rates that had evolved from the beginning of research until now. These decreasing performances with time have largely contributed to the misunderstanding of aerobic denitrification. It is however obvious that aerobic denitrification is responsible to a great and consequently non-negligible extent for the loss of nitrogen oxides in aerobic/anoxic environments and this reaction has to be taken into account in the nitrogen balance calculation in soils or wastewater treatment processes.

In order to go deeper into the physiology of aerobic denitrification, some experiments were conducted with variable relative proportions of the two electron acceptors and with variable concentrations of the electron donor. In fact, the two final respiratory acceptors may compete for the electron flux provided by carbon. By bubbling the medium with a mixture increasingly concentrated in oxygen, the increased pool of oxygen molecules could favor the diversion of the electron flux to oxygen instead of nitrate. In fact, the part of the electron flux to nitrate always remained constant between 7–0.35 mg DO l^{-1} for the consortium and 7–4.5 mg l^{-1} for the pure strain. This implied that the DO concentrations had no influence on the aerobic denitrifying activity of the consortium and of *M. aerodenitrificans* above a defined threshold. These observations are opposite to what

Table 5 Nitrogen-removal rates of the common and new metabolic pathways: nitrification, denitrification, Anammox, anaerobic nitrification-denitrification, aerobic denitrification and combined aerobic nitrification-denitrification systems. References: (1) Martins dos Santos et al. 1998, (2) Schmidt and Bock 1997, (3) Bode et al. 1987, (4) Jetten et al. 1999, (5) Patureau et al. 1996b, (6) Kuai and Verstraete 1998 and (7) Patureau et al. 1997. *N* Nitrification, *D* denitrification

Pathways	Nitrogen-removal rates (kg kg^{-1} dry weight day^{-1})	Nitrogen-removal rates (kg N m^{-3} day^{-1})	Nitrogen loads (kg N m^{-3} day^{-1})
Nitrification (1)			
Trickling filter	0.04–0.08	0.4–0.8	
Upflow filter	0.04–0.14	0.4–1.4	
Downflow filter	0.03–0.18	0.1–1.2	
Anaerobic reduction of NO_x by nitrifiers (2)	1.4×10^{-5}–0.002		
Denitrification (1)			
Upflow filter	0.08–0.3	0.8–3.0	
Fluidized bed	0.06–0.17	2.0–0.6	
Concentrated nitrate wastewater (3)	0.012 to 0.32		
Anammox (4)	0.05–0.26	0.42 to 2.45	0.46 to 2.63
Aerobic denitrification with the consortium (this work)	0.1–0.48	0.122–0.18	0.16–0.29
Aerobic denitrification with *M. aerodenitrificans*			
Continuous (DO = 33 mg l^{-1}; 5)	0.18–0.32		0.2
Continuous (DO = 7; this work)	0.13–1.75	0.066–0.35	0.16–1.4
Nitrification and denitrification by autotrophic nitrifiers (6)	0.016	0.052	0.13
Activated sludge ($N+D$; 1)	0.06–0.18	0.2–0.6	
Nitrifying ecosystem combined with *M. aerodenitrificans* under continuous culture (7)	0.083	0.009–0.027	0.0446

has been observed until now (Hochstein et al. 1984). These differing results suggest a characteristic feature of aerobic denitrification. It was first shown that the denitrifying enzymatic system and the oxygen respiration system function in parallel, which implyies that oxygen is not a direct inhibitor of the activity and of the synthesis of the denitrifying enzymes. However, as soon as the oxygen pool decreases, the denitrifying enzymes become increasingly active. This sensibility threshold towards oxygen for the synthesis and activity of the denitrifying enzymes was well illustrated by works on classical denitrifiers like *Pseudomonas stutzeri* (Körner and Zumft 1989) and *Rhodobacter sphaeroides* (Sabaty et al. 1994). Indeed, synthesis and activity of the *P. stutzeri* nitrate reductase started at 5 mg DO l^{-1} and 3.5 mg DO l^{-1} respectively. The main difference with *P. stutzeri* or *R. sphaeroides* is that the overall denitrifying enzymatic system of *M. aerodenitrificans* is active and thus synthesized between 7 mg l^{-1} and 4.5 mg l^{-1}. Moreover, it was shown that the latter co-utilized oxygen and nitrate, but the two other strains did not. As demonstrated a few years ago, no major differences were noticed between anoxic and aerobic enzymes and solely the nitrate reductase was doubled (Bell et al. 1990; Patureau 1995; Kobayashi et al. 1997). It seems that the difference between aerobic and anoxic conditions is the level of synthesis and activity. Improved aerobic denitrifying activity was noticed just after an anoxic period. It reflects the expression of the enzyme pool synthesized under anoxic conditions, maintaining a high level of activity to reach the level of aerobic expression and activity of the denitrifying enzymes.

Improvement of aerobic denitrifying activity was also noticed when the concentration of the secondary electron acceptor was increased together with the electron flux. Considering kinetic aspects (Monod denitrification rate expression), the aerobic denitrifying system operates in the same way as an anoxic one. It seems too that aerobic denitrification is electron flux-dependent, implying that the redox state of the cells or particular respiring proteins (redox-sensing factors) provide the regulatory framework for aerobic denitrification. However, the higher sensibility or reactivity of the aerobic denitrifying enzymatic system to moderate but decreasing electron flux modification shows that co-respiration can only be considered as a secondary pathway: under stress conditions, oxygen respiration dominates.

Thus it is obvious to properly define the growth conditions in order to obtain the best aerobic denitrifying rates. These remarks have implications for the design of any new aerobic system for the removal of nitrogen using nitrification and denitrification in combination. For example, the behavior of the consortium or *M. aerodenitrificans* toward oxygen can be useful for optimizing the combined system. Indeed, it was assumed that 100% nitrification could be obtained, up to DO concentrations of 3 mg l^{-1}. In the case of bioaugmentation of the nitrifying biomass with *M. aerodenitrificans*, working around 3 mg l^{-1} could enhance the global nitrogen-removal rate. However, working with a consortium gives a better advantage in terms of performance, but not in terms of oxygen-sensibility threshold.

To conclude, aerobic denitrification has serious implications on wastewater treatment processes as well as on natural ecosystems. Because most of the conventional nitrogen-removal systems are based on either the recirculation of biomass between aerobic and anoxic zones, or the application of alternate aerobic and anoxic conditions, the existence of this flexible nitrate–oxygen metabolism contributes to rapid acclimatization and effective response with non-negligible nitrate-removal rates. It has however a positive effect as long as the reaction is complete. It is thus necessary to quantify the possible unwanted emissions of nitric or nitrous oxides by incomplete aerobic denitrification. To prevent such problems, it is first essential to obtain more physiological and kinetic insight both into the mechanisms of action and regulation of the enzymes implied and into the environmental factors affecting their function. The consortium isolated here can be considered as a good support for strain characterization and as a good example for defining the tight operating conditions for a nitrification-denitrification system.

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