Nitrification-Denitrification in WSP: A mechanism for permanent nitrogen removal in maturation ponds

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Abstract
A pilot-scale primary maturation pond was spiked with ^{15}N-labelled ammonia ($^{15}NH_4Cl$) and ^{15}N-labelled nitrite ($Na^{15}NO_2$), in order to improve current understanding of the dynamics of inorganic nitrogen transformations and removal in WSP systems. Stable isotope analysis of $\delta^{15}N$ showed that nitrification could be considered as an intermediate step in WSP, which is masked by simultaneous denitrification, under conditions of low algal activity. Molecular microbiology analysis showed that denitrification can be considered a feasible mechanism for permanent nitrogen removal in WSP, which may be supported either by ammonia-oxidising bacteria (AOB) or by methanotrophs, in addition to nitrite-oxidising bacteria (NOB). However, the relative supremacy of the denitrification process over other nitrogen removal mechanisms (e.g., biological uptake) depends upon phytoplanktonic activity.

Keywords
^{15}N stable isotopes; denitrification; maturation pond; nitrification; nitrogen removal; tracer experiments

INTRODUCTION

Waste stabilisation ponds (WSP) are not normally considered a reliable technical option for nutrient removal from domestic wastewater; however, studies on WSP in the UK have shown that nitrogen is removed to low levels (<5 mg ammonium N per litre) in both winter and summer (Abis and Mara, 2003). Unfortunately current evidence is far from being able to determine which mechanism(s) dominate(s) nitrogen removal in WSP and, most importantly, under which operational and environmental conditions. Feasible transformation pathways and removal mechanisms for nitrogen control in domestic wastewater treatment by WSP could include: (a) ammonia volatilisation, (b) biological nitrogen uptake, (c) nitrification, (d) denitrification, and (e) sedimentation of dead biomass and accumulation in the sludge layer (Craggs, 2005), although nitrogen removal in WSP systems has been mainly attributed to ammonia volatilisation and sedimentation of organic nitrogen (Pearson, 2005). However, recent studies have demonstrated that under favourable conditions for algal growth, ammonium nitrogen is primarily removed by algal uptake despite high in-pond pH values (Camargo Valero and Mara, 2007a). That leads to high ammonium removal rates but low total nitrogen removals, as much of the ammonium taken up by the algae leaves the pond in its effluent as suspended solids. Therefore, it would be expected that other mechanisms such as simultaneous nitrification-denitrification might play an important role on permanent nitrogen removal in WSP, especially as ammonia volatilisation has been shown not to make any significant contribution to total nitrogen removal (Camargo Valero and Mara, 2007b).

In view of the low nitrate and nitrite concentrations in WSP, it has been suggested that nitrification is not likely to occur in maturation ponds despite prevalent in-pond aerobic conditions and long retention times. However, it has been demonstrated that nitrifiers can grow in WSP (up to 10^7 organisms/ml in in-pond water samples; Morrison, 1984), and also that high concentrations of nitrite and/or nitrate (up to 6 mg N/l), which usually correspond with high ammonium nitrogen removals, can be found in pond effluents (Santos and Oliveira, 1987; Hurse and Connor, 1999). Simultaneous processes such as biological nitrate uptake

and/or denitrification would help to explain the apparent absence of nitrification in WSP. Camargo Valero and Mara (2007c) found that the nitrification process was masked by simultaneous algal nitrate uptake during the peak of algal activity in a maturation pond in the UK. On the other hand, simultaneous nitrification-denitrification has been reported as the main mechanism for permanent nitrogen removal in WSP (e.g., Lai and Lam, 1997; Zimmo *et al*., 2004; Picot *et al*., 2005; Strang and Wareham, 2005), although hardly any evidence regarding to nitrogen transformation pathways dominating nitrification and denitrification has been reported.

Most of the research conducted to elucidate the main nitrogen removal mechanisms in WSP have been based on the measurements of nitrogen fractions (organic, ammonium, nitrite and nitrate) in water samples collected from both the pond influent and effluent. However, such approaches may in fact render any understanding of the fate (or fates) of nitrogen in WSP particularly difficult in situations in which water quality changes are so small that they do give any evidence about simultaneous processes (e.g., nitrification-denitrification, nitrification-biological nitrate uptake). A much better approach to further our understanding of the fate(s) of nitrogen compounds in WSP is based on the use of stable nitrogen isotopes (^{15}N), which have been largely used to illustrate the behaviour of nitrogen in aquatic ecosystems and, more recently, in wastewater treatment units. In addition, little work has been done in WSP to relate the fate of nitrogen compounds to the prevailing biology of the system. In this work, tracer experiments using ^{15}N stable isotopes, along with molecular microbiological analyses, were carried out in a pilot-scale maturation pond in the UK to facilitate the study of the dynamics of inorganic forms of nitrogen under conditions of low algal activity, in order to determine the relative importance of nitrogen transformations and removal mechanisms associated with nitrification and denitrification processes.

METHODS AND MATERIALS

This research was undertaken on an experimental pilot-scale WSP system at Esholt Wastewater Treatment Works in Bradford, West Yorkshire, UK. The pilot-scale WSP system comprises one primary facultative pond (PFP) fed with screened wastewater (50% domestic, 50% industrial), two maturation ponds in series (M1 and M2), and a reedbed channel (RBC). The PFP was loaded at 80 kg BOD/ha d (8 g BOD/m^2 d) and 8 kg N/ha d (0.8 g N/m^2 d), with an average nominal retention time (θ_0) of 60 days within the experimental timeframe reported herein. Pond M1 (6.3 × 3.5 × 1.00 m) received effluent from the PFP which was pumped at an average rate of 0.6 m^3/d (θ_0 = 17.5 d); the effluent from M1 discharged by gravity into M2 and thence also by gravity into the RBC.

Tracer experiments with ^{15}N-labelled ammonium and nitrite salts were conducted in pond M1 in winter 2006–07. M1 was spiked firstly with a single pulse of $^{15}NH_4Cl$ (0.7 g) and after $3\times\theta_0$ with $Na^{15}NO_2$ (1.0 g) (the ^{15}N salts (98% ^{15}N) were supplied by Cambridge Isotope Laboratories, Cambridge, USA). M1 effluent was sampled hourly for $1\times\theta_0$ before the first spiking and for $6\times\theta_0$ afterwards by using an auto-sampler (Aquacell P2-Multiform; Aquamatic, Manchester, England). Samples were preserved in situ by the addition of 5 ml of preservative solution (6N HCl containing 2 g $CuCl_2$/l) per litre of sample. Simultaneously, a multi-parameter sonde probe (model YSI 6820; YSI Inc., Yellow Springs, USA) was used to measure in real time dissolved oxygen (DO), temperature and pH in the M1 effluent.

Collected samples were taken to the Public Health Laboratory, University of Leeds, where 24-hour composite samples were made. The composite samples were processed for ammonium (method 4500-NH3 B; APHA, 1998), suspended solids (SS) (2540 D), TKN and filtered TKN (4500-Norg C), and nitrite and nitrate by ion chromatography (IC-ED; DX500, Dionex Cop., Sunnyvale, USA), following the analytical procedure described by Raessler and Hilke (2006). Samples were also sequentially partitioned to extract four nitrogen species separately: (a) suspended organic nitrogen, by filtering on pre-ashed (550ºC) fibre-glass filters (GF/C; Whatman International Ltd, Maidstone, England); (b) soluble organic nitrogen, by solid phase extraction (Isolute C18 cartridge; Biotage, Uppsala, Sweden), followed by elution with absolute ethanol and further concentration on pre-ashed fibre-glass filters (Whatman GF/D) by volatilization at 40ºC; (c) ammonium nitrogen, by ammonia diffusion (Holmes *et*

al., 1998); and (d) oxidised nitrogen, by nitrate and nitrite reduction into ammonium with Devarda's alloy (Brooks *et al.*, 1989) and simultaneous ammonia extraction by diffusion (Holmes *et al.*, 1998). Each fraction was analyzed to determine ^{15}N:^{14}N ratios using an elemental analyzer coupled with a stable isotope ratio mass spectrophotometer (EA-IRMS; EuroEA3000-Micromass Isoprime, Eurovector, Milan).

Ammonia losses by volatilization were estimated on site following the procedure described by Camargo Valero and Mara (2007b); samples were processed for ammonium and ^{15}N:^{14}N ratios, as described above. Settled organic nitrogen samples were collected in 10-litre metal buckets which were strategically placed on the bottom of M1 and taken out at the end of each experiment. Collected sediment samples were sieved (ASTM sieve No. 10) to remove coarse solids and settled in 1-litre Imhoff cones for 3 hours. Thickened samples were dried at 105ºC and processed simultaneously for nitrogen content and ^{15}N:^{14}N ratios. Sediment sub-samples were also processed for solids and moisture content (2540 B, 2540 D, 2540 F). Additionally, a weekly sampling for performance indicators was carried out by determining on site temperature, DO and pH and collecting samples from the M1 influent, water column and effluent. These samples were analyzed for BOD_5 and filtered BOD_5 (5210 B), chlorophyll *a* (Pearson *et al*., 1987) and SS, TKN, filtered TKN, ammonia, nitrate and nitrate, as described previously.

Table 1. The primers used for the PCR detection and analysis of nitrogen-transforming microbes.

Primer pair sequence (5' to 3')	Primer set common name	Target gene	Target functional group/organisms	References
GGAGRAAAGYAGGGGATCG CTAGCYTTGTAGTTTCAAACGC	CTO189f-CTO654r	Bacterial *amoA*, ammonia mono-oxygenase	ß-proteobacterial ammonia oxidizing bacteria (AOB)	Kowalchuk *et al.* (1999), Rowan *et al.* (2003)
STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	Arch-AmoAf-Arch-AmoAr	Crenarchaeotal *amoA*, ammonia mono-oxygenase	Ammonia-oxidizing archaea (AOA)	Francis *et al.* (2005)*
GACTTGCATGCCTAATCC CCTTTCGGGCATTGCGAA	Pla46-Amx368	16S ribosomal RNA	Anammox bacteria	Schmid *et al.* (2005)*[a]
GGNGACTGGGACTTCTGG GAASGCNGAGAAGAASGC	pmoA189f-pmoA682r	*pmoA*, particulate methane-monoxygenase, *amoA*, ammonia-monooxygenase	Most methanotrophs and some ammonia-oxidizing bacteria	Holmes *et al.* (1995), Dunfield *et al.* (1999)
GGNGACTGGGACTTCTGG CCGGMGCAACGTCYTTACC	pmoA189f-mb661r	*pmoA*, particulate methane-monoxygenase	Most methanotrophs	Costello and Lidstrom (1999)*
AGAGTTTGATCMTGGCTCAG GGCCTTCYTCCCGAT	Bact27f-Nspa705r	16S ribosomal RNA	*Nitrospira* nitrite-oxidizing bacteria	Freitag *et al.* (2005)
AGAGTTTGATCMTGGCTCAG CACCTGTGCTCCATGCTCCG	Bact27f-Nbac1050r	16S ribosomal RNA	*Nitrobacter* nitrite-oxidizing bacteria	Freitag *et al.* (2005)
GTSAACGTSAAGGARACSGGG ASTTCGGRTGSGTC TTGA	Cd3aF-R3cd	*nirS*, cytochrome cd_1 nitrite reductase	Denitrifiers	Throbäck *et al.* (2004)
ATCATGGTSCTGCCGCG GCCTCGATCAGRTTGTGGTT	F1aCu-R3Cu	*nirK*, Cu-containing nitrite reductase	Denitrifiers	Throbäck *et al.*, 2004

[a] PCR conditions as reference using 39 cycles during the annealing step

* the forward primer contained the following GC-clamp in DGGE; CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGG

For molecular microbiological analyses, representative samples for winter conditions were collected for molecular analysis from the sludge layer and the M1 water column (at 0.10, 0.45 and 0.85 m depths); they were preserved with absolute ethanol (1:1 v/v) and stored at –20ºC in the laboratory before being processed. In addition, some of the samples from different depths in the water column were made into composite samples. Total genomic DNA was extracted from each sample by using the FastDNA kit for soils as described in the manufacturers' instructions (Q-Biogene, MP Biomedicals, UK). The 16S rRNA gene or functional gene fragments of bacterial groups specifically involved in nitrogen transformations were targeted by PCR using previously published primers and conditions (Table 1). PCR was used to confirm the presence or absence of different microbial groups, and selected PCR-positive samples were further analysed to confirm the identity of microorganisms putatively belonging to those groups. Microbial community analysis was performed using the community fingerprinting method, denaturing gradient gel electrophoresis (DGGE; e.g. Rowan *et al.,* 2003), as previously described for respective groups (Table 1), with the excision, clean-up and sequencing of selected predominant bands (e.g. Milner *et al.,* 2008). The subsequent sequences were checked against the public database repository, GenBank (Benson *et al.,* 2008), using the BLAST tool to identify the closest matching sequence/organism, and/or classified using the RDP classifier tool (Wang *et al.,* 2007). Corresponding microbial analyses were interpreted together with the ^{15}N tracer experiment results.

RESULTS AND DISCUSSION

Data from the ^{15}N tracer experiments were collected for $6\times\theta_0$ in total; hence the ^{15}N:^{14}N ratio values in the M1 effluent were solely influenced by the addition of ^{15}N-labelled ammonia during the first half of this experiment ($0< t/\theta_0< 3$), whist in the second half ($3< t/\theta_0< 6$), they were mainly affected by the ^{15}N-labelled nitrite spike. The results for ^{15}N:^{14}N ratios from samples collected in M1 effluent are reported as delta values in parts per thousand ($\delta^{15}N$, ‰). $\delta^{15}N$ values are not concentrations of the ^{15}N isotope but differences between ^{15}N:^{14}N ratios in the sample ($^{15}N/^{14}N_{spl}$) and atmospheric N_2 gas ($^{15}N/^{14}N_{std}$), which has a known ^{15}N content and acts as a standard. Instrument calibration was done with two certified standards of labelled ammonium sulphate: IAEA-USGS26 ($\delta^{15}N = +53.7$) and IAEA-USGS25 ($\delta^{15}N = -30.4$), provided by the U.S. Geological Service (Denver, CO) and certified by the Section of Isotope Hydrology, International Atomic Energy Agency (Vienna). The standard error in $\delta^{15}N$ readings of the certified standards was ±0.12‰ at most. The reported $\delta^{15}N$ values have been corrected for background content based on results from samples collected before tracer injection (^{15}N baseline); therefore negative values are graphed as zero as they include only tracer ^{15}N.

After ^{15}N-labelled ammonium was injected into M1 (Figure 1), the ammonium nitrogen fraction was highly enriched with ^{15}N, as expected, and it decayed slowly within the first half of the sampling period ($0< t/\theta_0< 3$). Nitrogen fractions in M1 effluent were enriched with ^{15}N as follows: ammonium fraction, ~730‰; oxidised fraction, ~420‰; suspended organic fraction, ~60‰; and soluble organic fraction, ~50‰. Therefore, it seems that ammonium oxidation to nitrite and nitrate was the preferred transformation pathway, followed by biological uptake. It is important to highlight that labelled ammonium was mainly washed out the system as nearly a half of the injected tracer was recovered in the pond effluent as $^{15}NH_4^+$ (Table 2). The ^{15}N mass balance showed that over a period of time equal to $3\times\theta$, nearly 10.0 percent of the tracer could not be accounted in any of the nitrogen fractions in M1 effluent or remaining inside M1 pond.

M1 was spiked with ^{15}N-labelled ammonium during the coldest period in winter 2006-07, when the water temperature was between 3.1 and 6.4ºC. Low water temperatures, a short photoperiod (7.8−10.8 daylight hours per day) and few sunlight hours per day (2.4 hours on average) were responsible for a very low photosynthetic activity in the M1 pond, which had a mean water-column chlorophyll *a* concentration of only 46 µg/l ($6.1<pH<7.6$). Despite these seemingly adverse conditions, total nitrogen removal in M1 was 813 g N/ha d (27%), which is higher than the removal reported from the same pond in summer (~ 8%; Camargo Valero and Mara, 2007c). Moreover, ammonium removal was negligible as there was no significant difference when mean ammonia values from M1 influent and effluent were compared by using the *t*-test [$t\,(10) = -1.232$; $p = 0.234$].

Performance indicators from the weekly sampling conducted during the first spike ($0 < t/\theta_0 < 3$) showed that M1 received loadings of 6.2 kg BOD/ha d (0.6 g BOD/m^2 d) and 3.0 kg N/ha d (0.3 g N/m^2 d). SS and suspended organic nitrogen removals were 71 and 81 percent, respectively. It could be expected that sedimentation of the organic nitrogen fraction present in M1 influent was the main mechanism for total nitrogen removal in winter; nevertheless, the corresponding nitrogen sedimentation rate (228 g N/ha d) contributed only a quarter of the total nitrogen removal rate. Ammonia volatilisation rate was negligible, as expected (0–2 g N/ha d), although the collected ex-pond gases where enriched with ^{15}N from 16.17 to 35.77‰ when compared with the corresponding baseline (from −42.40 to −31.10‰).

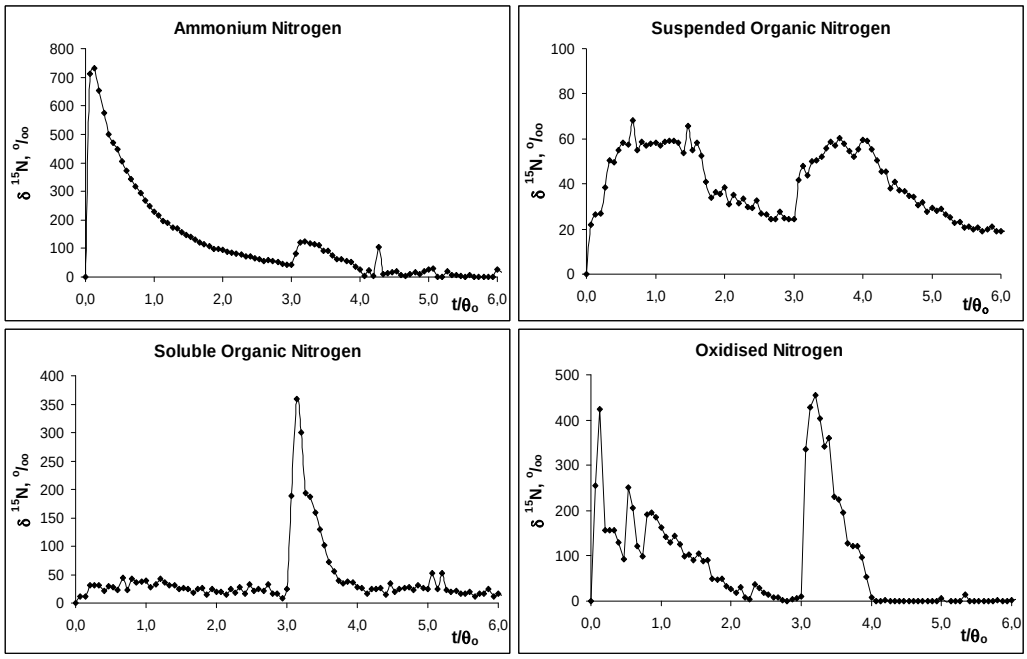

Figure 1. $\delta^{15}N$ values in nitrogen fractions from M1 effluent after spiking

Table 2. Cumulative ^{15}N-labelled recovery during tracer experiments

Nitrogen fractions	Tracer recovery, %	
	* $^{15}NH_4^+$	** $^{15}NO_2^-$
Recovered in M1 effluent		
Suspended organic nitrogen	2.0	3.3
Soluble organic nitrogen	0.8	4.8
Ammonium	55.7	1.9
Nitrite + Nitrate	3.0	1.5
Remaining in water column	~4.0	~1.5
Stored in sludge layer	~24.5	~30.0
Ammonia volatilisation	0.0	0.0
Net recovery	~90	~43.0

* The mass balance was calculated over the $0 < t/\theta_0 < 3$ period
** The mass balance was calculated over the $3 < t/\theta_0 < 6$ period

M1 was also spiked with $Na^{15}NO_2$ in order to elucidate the fate of oxidised forms of nitrogen in WSP; the corresponding $\delta^{15}N$ values are shown in Figure 1 ($3 < t/\theta_0 < 6$). Taking into account that each tracer experiment was run for about 50 days ($3\times\theta_0$), the weather conditions for this tracer experiment (late winter – early spring) were different from those during the earlier tracer run with ^{15}N-labelled ammonium. The water temperature ranged from 5 to 12ºC, and daylight was between 10.8 and 13.9 h/d

(5.3 mean sun hours per day) and consequently the mean in-pond values for photosynthesis-associated parameters, such as chlorophyll *a* (250 μg/l), pH (6.8–8.2) and DO (5.3 mg/l), were more favourable for algal ammonium uptake. In fact mean ammonium nitrogen removal during this experiment was 75 percent and mean total nitrogen removal 18 percent. Ammonia volatilisation was also negligible during this experiment. M1 received loadings of 6.6 kg BOD/ha d (0.7 g BOD/m^2 d) and 1.8 kg N/ha d (0.2 g N/m^2 d).

Labelled nitrite was rapidly transformed immediately after the tracer injection as it can be appreciated from the oxidised nitrogen fraction (Figure 1) which was enriched with ^{15}N up to 450‰ and decreased almost completely in only $1\times\theta_0$. The ammonium nitrogen fraction was also enriched (~120‰), as was the suspended organic nitrogen fraction (~60‰), but surprisingly the soluble organic nitrogen increased up to ~360‰. That could be associated with biochemical transformations of labelled nitrite intermediated by enzymes which could have been extracted on the soluble organic fraction and therefore it would explain the considerable increment of ^{15}N in that fraction immediately after the tracer was injected. Results from a ^{15}N mass balance for $3\times\theta_0$ (Table 2) showed that the labelled nitrite tracer was poorly recovered in the M1 effluent (11.5%). The tracer mass balance also found a large accumulation in the sludge layer (~30.0%) but a very small one in the water column (~1.5%); the remaining ^{15}N (~43.0%) could not be accounted for in any of these fractions.

Considering the ^{15}N enrichment in samples collected from ammonia volatilisation experiments carried out during the previous tracer spike with ^{15}N-labelled ammonium, it was decided to change the 2% boric acid solution in the ammonia absorption system (Camargo Valero and Mara, 2007b) to a 1% sodium hydroxide solution during the second tracer spike with ^{15}N-labelled nitrite. It would increase the capacity of the system to absorb acid gases coming out from M1 pond, such as NO_x. Effectively, $\delta^{15}N$ values from collected samples were ranged from 10.05 to 52.79‰, confirming that there were ^{15}N-labelled gases with acid characteristics leaving M1 through the pond surface to the atmosphere (e.g., NO, N_2O); this is in agreement with N_2O emissions detected in gas samples collected from maturation ponds in France (Picot *et al*., 2007).

Tracer experiments with ^{15}N-labelled ammonium and nitrite showed that nitrification was masked by simultaneous denitrification. This is based on the following observations: (a) ^{15}N-labelled ammonium was oxidised to nitrite and nitrate; (b) there was no substantial nitrate accumulation in M1 effluent; (c) a cumulative ^{15}N mass balance showed that the tracers ($^{15}NH_4^+$ and $^{15}NO_2^-$) were not completely recovered after a $3\times\theta_0$ time period (90% and 57% recovery, respectively); (d) nitrogen losses via ammonia volatilisation was negligible; and (e) gases leaving the maturation pond to the atmosphere were clearly enriched with ^{15}N during both tracer experiments. Therefore, simultaneous nitrification-denitrification may be responsible for most of the nitrogen permanently removed from the maturation pond M1 during this experimental timeframe (winter – early spring).

Table 3. Results for the presence/absence of microbial groups by PCR

Sample	Ammonia-oxidizers		Nitrite-oxidizing bacteria		Anammox	Methanotrophs	Denitrifiers	
	AOB	AOA	*Nitrobacter*	*Nitrospira*			*nirS*	*nirK*
Water column	+	±	+	+	–	+	+	+
Sludge	+	–	+	+	–	+	+	+

Key: + = strong band detected, ± = weak band detected, - = no band detected; AOB, ammonia-oxidizing bacteria; AOA, ammonia-oxidizing archaea.

Results from the molecular microbial analyses carried out on samples from the maturation pond M1 (Table 3) are consistent with the above thesis. PCR revealed the presence of microorganisms that would be capable of classical nitrification and denitrification including; ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), methanotophs, nitrite-oxidizing bacteria, and denitrifiers. In contrast, anaerobic ammonia oxidation, anammox, could be discounted since the microorganisms

capable of this process were not detected by PCR. The first step in the classical pathway for nitrification of ammonia oxidation by AOB has recently been challenged by the detection of archaeal amoA genes in marine and terrestrial environments (Francis *et al*., 2005; Prosser and Nicol, 2008). Few have so far been detected in freshwater environments (Park *et al.,* 2006). A weak band was detected using AOA-specific primers, the resultant weak DGGE band sequence from which was confirmed to closely match an uncultured crenarchaeote clone (98% similarity, BLAST). The clear bands obtained by AOB-specific PCR and DGGE would indicate that AOB were more predominant, although confirmation of the identity is still required. These organisms are also capable of denitrification in anoxic and low-oxygen environments (Schmidt *et al*., 2002, 2003; Kampschreur *et al.,* 2006), conditions which can be prevalent in the M1 pond (e.g., winter conditions).

On the other hand, methanotrophs were detected in the water column and sludge samples, and although confirmatory DGGE of these samples were not performed, confirmed methanotroph-like sequences were obtained from the effluent of the M1 maturation pond. Considering that the co-metabolism of ammonium by methane-oxidising bacteria as methane mono-oxygenase is very similar to ammonium mono-oxygenase, methanotrophs can also catalyze the oxidation of ammonium (nitrification) and produce nitric and nitrous oxides (Murrell and Radajewski, 2000). Although methanotrophs are not themselves known to carry out denitrification, there is good evidence that denitrifying bacteria can be associated with methanotrophs and can use simple carbon compounds released by the methanotrophs as substrates for the denitrification reactions and for growth (Knowles, 2005). Such denitrification associated with methanotrophs can release nitrogen gases (e.g., NO, N_2O, N_2) and so contribute to permanent nitrogen removal from WSP. At this point, it is important to mention that methanotrophs require methane as carbon source and, although it is believed that methanogenesis does not occur below 13ºC, it has been found that methanogens consistently exposed to low temperatures maintain their activity (Pearson, 2005; Juanico *et al*., 2000).

Nitrite-oxidising bacteria (NOB) were also detected in all samples analysed. Analysis of NOB-specific DGGE bands revealed the presence of multiple sequences classified as *Nitrospira* (80% confidence, RDP classifier) but no *Nitrobacter*. These results indicate that the full classical aerobic nitrification pathway is possible in the M1 pond. Moreover, results targeting the *nirS* and *nirK* genes revealed the presence of putative denitrifiers (99% and 89% similarities respectively with uncultured clones) in M1 pond. Therefore denitrification supported either by AOB or methanotrophs, in addition to NOB, in WSP may be counted as a feasible mechanism for permanent nitrogen removal, but its relative supremacy over other nitrogen removal mechanisms (e.g., biological uptake) would depend upon phytoplanktonic activity.

Nitrate and nitrite concentrations do not increase in pond effluents because nitrification may be masked by simultaneous biochemical reactions such as biological nitrate uptake and/or denitrification; therefore, nitrification can definitely be considered as an intermediate step in nitrogen transformation and removal in WSP. A net accumulation of oxidised nitrogen species (nitrite and nitrate) could be expected in the pond effluent, if environmental and operation conditions in WSP system were less favourable for algal growth and for the performance of the denitrification process. Such conditions could be found in the final treatment units of a WSP system with a large number of ponds connected in series. The final ponds would receive lower ammonium and biodegradable organic matter (soluble BOD) loadings – ammonium is important for algal growth, whilst soluble BOD is essential as an organic carbon source for denitrification. In fact, a study case reported by Lai and Lam (1997) could help to consolidate this hypothesis: they found a net increment of nitrate and nitrite concentrations from samples collected in the effluent of the final ponds of a WSP system with eight ponds in series (Melbourne, Australia) during late autumn and winter.

CONCLUSIONS

Tracer experiments with ^{15}N stable isotopes showed a clear competition for inorganic nitrogen species between the two main mechanisms dominating nitrogen removal in maturation ponds: algal uptake and nitrification-denitrification. In fact, when environmental conditions were not favourable for algal growth (winter), ammonium nitrogen was mainly transformed into oxidised nitrogen species and then permanently removed via the denitrification process. On the other hand, when environmental conditions were more favourable for phytoplanktonic activity (late winter – early spring), ammonium nitrogen was removed more efficiently by algal uptake and simultaneously removed by nitrification-denitrification. The results reported in this research work, which included both ^{15}N tracer experiments and molecular microbiology analyses, are new evidence to support nitrification-denitrification being one of the two major mechanisms for permanent nitrogen removal in WSP, along with sedimentation of dead algal biomass after biological nitrogen uptake.

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