8. RESULTS AND DISCUSSION: TRACER EXPERIMENTS WITH ^{15}N STABLE ISOTOPES IN MATURATION PONDS

The mechanisms and pathways by which nitrogen in its various forms is removed from WSP have been a subject of much debate for wastewater scientists and engineers. In order to improve current understanding of the dynamics of inorganic and organic nitrogen removal in WSP systems, a study using stable nitrogen isotopes (^{15}N) was undertaken as part of this research project. The M1 maturation pond was spiked separately with ^{15}N-labelled ammonia ($^{15}NH_4Cl$), ^{15}N-labelled nitrite ($Na^{15}NO_2$) and ^{15}N-labelled algae (*Chlorella vulgaris*) to track the fate of organic and inorganic nitrogen species in WSP. The results reported in this chapter are aimed to reveal the relative importance of the nitrogen transformations and removal associated with ammonia volatilisation, nitrification, and algal uptake and its subsequent sedimentation and retention/hydrolysis in the sludge layer.

8.1 Reporting Results for ^{15}N Tracer Experiments

The results for 15N:14N ratios from samples collected in M1 effluent are reported as delta values in parts per thousand ($\delta^{15}N$, ‰) (equation 2.17). In fact, they are not concentrations of the ^{15}N isotope but differences between 15N:14N ratios in the sample and atmospheric N_2 gas as mentioned previously (section 2.1.5). Instrument calibration was carried out 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.

Composite samples collected from M1 effluent were sequentially partitioned as described previously (section 3.6.2) and the corresponding fractions were processed for 15N:14N ratios. In this chapter, the reported $\delta^{15}N$ values for suspended organic nitrogen, soluble organic nitrogen, ammonium nitrogen and oxidised nitrogen (nitrite and nitrate) were corrected for background content based on results from samples collected before tracer injection (^{15}N baseline); therefore negative values are reported as zero as they include only tracer ^{15}N.

8.2 Tracer Experiment with ^{15}N Stable Isotopes in Summer

Two tracer runs were carried out with ^{15}N stable isotopes under summer conditions. The first tracer spike was done with ^{15}N-labelled ammonia ($^{15}NH_4Cl$) in summer 2005 and the second with ^{15}N-labelled algae (*Chlorella vulgaris*) in summer 2006.

8.2.1 Tracer run with ^{15}N-labelled ammonium

After the tracer was injected into M1 in summer 2005 (Figure 8.1), the ammonium nitrogen fraction was highly enriched with ^{15}N, as expected, but it decayed rapidly within the experimental timeframe. This may mean that ^{15}N-labelled ammonium was mixed rapidly in the pond and also that it was involved in a process with a very high reaction rate. Ammonia volatilisation cannot be considered to be the dominant mechanism for ammonium removal (see chapter 6), even though temperature (15.2–18.2ºC) and pH (8.9–10.2) were very favourable for its occurrence. Ammonia volatilisation rates were found to be very low (<1–27 g N/ha d) and the corresponding $\delta^{15}N$ values (−42.40 to −31.10‰) were not significantly different from the background samples (p >0.05), which means that the small amount of volatilized ammonia was not substantially enriched with ^{15}N. Ammonium nitrogen was removed at 3,747 g N/ha d (90% ammonium removal) in summer 2005 and its 95-percentile concentration in M1 effluent was less than 2 mg N/l.

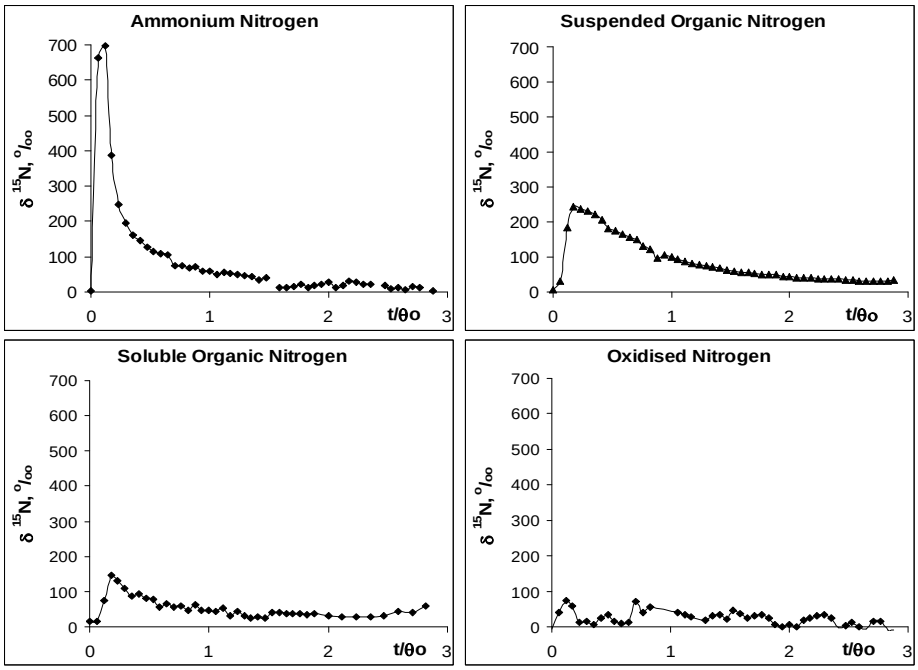


Figure 8.1 $\delta^{15}N$ values in nitrogen fractions from M1 effluent in summer 2005

The suspended organic fraction in M1 effluent was ~250‰ enriched with ^{15}N and considering that fraction increased from 3.5 to 13.4 mg N/l (Table 8.1) in comparison with M1 influent, biological (algal) uptake can be considered as the main mechanism for ammonium nitrogen removal. Performance indicators from the weekly sampling (Table 8.1) showed that M1 received loadings of 9.3 kg BOD/ha d and 6.0 kg N/ha d in summer 2005.

Table 8.1 Water quality results from weekly sampling in M1 pond: summer 2005

Parameter	Influent	Effluent
SS, mg/l	53 ± 12*	133 ± 7
BOD_5, mg O_2/l	30.0 ± 4.9	43.0 ± 4.0
Filtered BOD_5, mg O_2/l	10.9 ± 3.2	3.2 ± 0.7
TKN, mg N/l	17.4 ± 0.5	16.0 ± 0.4
Filtered TKN, mg N/l	13.9 ± 1.2	2.6 ± 0.5
NH_4^+, mg N/l	11.8 ± 1.3	1.5 ± 0.3
NO_3^-, mg N/l	0.06 ± 0.01	0.07 ± 0.02
NO_2^-, mg N/l	< 0.03	< 0.03
Chlorophyll *a*, μg/l	401 ± 169	1141 ± 57
E. coli, $\log_{10}$ (cfu/100ml)	5	4

* average ± standard error

Additionally, the organic fraction of samples collected from the sediments in M1 were enriched with ^{15}N (from +5.0 to +54.1‰, $\delta^{15}N$), which confirms that sedimentation of dead algal cells does contribute to total nitrogen removal. Decay of dead algal cells would have released not only ammonium but also soluble organic nitrogen which was highly enriched with ^{15}N in M1 effluent ($\delta^{15}N$ = ~130‰,). Therefore, organic nitrogen in the sludge layer is partially recycled to the water column as ammonium and soluble organic nitrogen compounds and it may be considered as an intermediate step in the nitrogen removal process in WSP.

Although oxidised forms of nitrogen like nitrate did not show any significant change when mean concentrations from M1 influent and effluent were compared (p >0.05), and nitrite concentrations were negligible during experimental timeframe (<0.03 mg N/l), this fraction was clearly enriched with ^{15}N (~80‰, $\delta^{15}N$ enrichment). Therefore, labelled ammonium was also transformed into oxidised nitrogen forms (nitrite and nitrate), thanks to in-pond oxidising conditions (mean DO = 11.0 mg/l). In fact, research carried out at the Werribee Treatment Complex (Melbourne, Australia) reported not only the presence of nitrifiers (10^7 organisms /ml) in in-pond water samples (Morrison, 1984), but also that high concentrations of nitrite and/or nitrate (up to 6 mg N/l) usually correspond with high

ammonium nitrogen removals (Hurse and Connor, 1999). Such evidence helps to show that nitrification does occur in WSP and that, despite low nitrite and nitrate concentrations in the pond effluent, nitrification may be considered as another intermediate step in nitrogen transformation and removal in WSP.

High nitrite concentrations are rarely found either in natural environments or in wastewater treatment plants, as with a sufficient oxygen supply nitrite oxidation to nitrate proceeds at a faster rate than the conversion of ammonium to nitrite (Schmid *et al*., 2003). On the other hand, nitrate concentration did not rise in pond effluent because nitrification may be restrained by the ammonium nitrogen availability or masked by biological uptake (or simultaneous denitrification). Algae and bacteria will commence the uptake of nitrate when ammonium becomes low (<1mM for bacteria and <1μM for algae – Magasanik, 1988; Flynn, 1991); however, nitrate should be firstly reduced to ammonium. In fact, nitrate will be reduced to ammonium only when ammonium is limiting as the presence of ammonium represses the action of the nitrate reductase system (Bagchi *et al.*, 1985).

Table 8.2 Cumulative* ^{15}N-labelled ammonium recovery in summer 2005

Nitrogen Fraction	**Recovery, %**
Recovered in M1 effluent	
Suspended organic nitrogen	48.9
Soluble organic nitrogen	4.9
Ammonium	8.8
Nitrite + Nitrate	0.2
Remaining in water column	~29.8
Stored in sludge layer	~7.4
Ammonia volatilisation	0.0
Net recovery	~ 100

* The mass balance was calculated over a $3\times\theta_0$ period.

Results for a ^{15}N mass balance are shown in Table 8.2. After $3\times\theta_0$, the ^{15}N tracer was mainly recovered in the M1 effluent (62.8%) as suspended organic nitrogen (48.9%), whilst ~37.2 percent was accumulated in the water column (~29.8%) and sludge layer (~7.4%). A nearly 100 percent recovery of the injected tracer reveals that total nitrogen removal was not lead by any mechanism involving nitrogen losses to the atmosphere (e.g., ammonia volatilisation, denitrification). Therefore, ammonium nitrogen was efficiently incorporated into suspended biomass (e.g., algal cells) and any net nitrogen removal was achieved by sedimentation of dead algal cells. It would also explain why total nitrogen

was removed in M1 only at 531 g N/ha d (8% total nitrogen removal), in comparison with ammonium removal (3,747 g N/ha d; 90% ammonium removal).

8.2.2 Tracer run with ^{15}N-labelled algae

The results from summer 2006 (Figure 8.2) confirmed a similar nitrogen isotope behaviour despite the ^{15}N source being organic nitrogen rather than inorganic nitrogen as in summer 2005. In this experiment dead cells of *Chlorella vulgaris* containing ^{15}N were rapidly hydrolyzed in the sludge layer, so releasing labelled organic compounds as an intermediate step to provide labelled ammonium nitrogen which was simultaneously transformed into nitrate and taken up by the biomass in water column.

The suspended organic nitrogen fraction (Figure 8.2), clearly shows the importance of nitrogen being recycled from sludge layer into the water column and how it is steadily supplying nitrogen for biological uptake. Again, ammonia removal by volatilisation was negligible (<1–15 g N/ha d) and organic nitrogen sedimentation was confirmed from sludge samples enriched with ^{15}N from +14.7 to +42.1‰ (δ^{15}N).

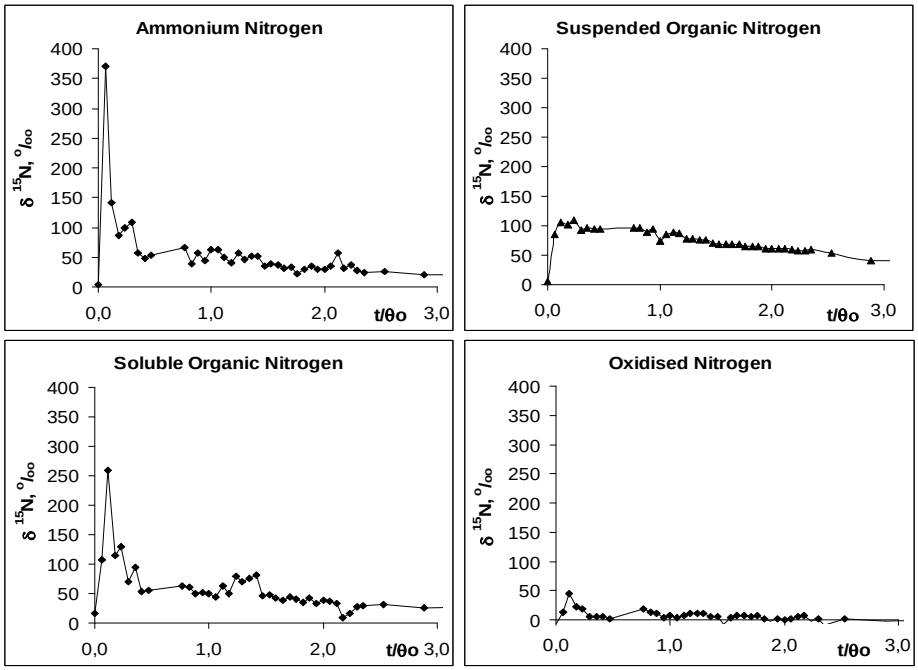


Figure 8.2 δ^{15}N values in nitrogen fractions from M1 effluent in summer 2006

Performance indicators from the weekly sampling (Table 8.3) showed that M1 received loadings of 7.6 kg BOD/ha d and 2.0 kg N/ha d. Ammonium nitrogen removal was only 40 percent and there was no significant difference between total nitrogen concentrations in the M1 inlet and outlet when mean values were compared by the *t*-test (t = 0.363, p =

0.723). In comparison with results from summer 2005, the mean in-pond values for photosynthesis-associated parameters, such as chlorophyll *a* (367 μg/l), pH (7.2–11.0) and DO (8.9 mg/l), were less favourable for algal nitrogen uptake, and this would explain the observed negligible total nitrogen removal and lower ammonium removal, as these processes appear to be dominated by photosynthetic activity during warm summer months.

Table 8.3 Water quality results from weekly sampling in M1 pond: summer 2006

Parameter	Influent	Effluent
SS, mg/l	45 ± 8	45 ± 4
BOD_5, mg O_2/l	21.5 ± 6.3	20.5 ± 3.3
Filtered BOD_5, mg O_2/l	8.3 ± 3.5	4.6 ± 0.9
TKN, mg N/l	5.8 ± 0.9	5.4 ± 0.6
Filtered TKN, mg N/l	2.8 ± 0.5	3.0 ± 0.4
NH_4^+, mg N/l	1.0 ± 0.4	0.6 ± 0.1
NO_3^-, mg N/l	0.12 ± 0.02	0.13 ± 0.05
NO_2^-, mg N/l	< 0.03	< 0.03
Chlorophyll *a*, μg/l	316 ± 110	334 ± 98
E. coli, $\log_{10}$ (cfu/100ml)	4	3

* average ± standard error

A ^{15}N mass balance for $3\times\theta_0$ showed that the tracer mainly accumulated (72.4%) in M1 water column and sludge layer (Table 8.4). Tracer recovery was again nearly 100 percent which confirms that nitrogen species are mainly transformed inside the maturation pond (e.g., ammonium to organic nitrogen) and that any nitrogen removal was achieved ultimately by biological uptake and further sedimentation.

Table 8.4 Cumulative* ^{15}N-labelled algae recovery in summer 2006

Nitrogen Fraction	Recovery, %
Recovered in M1 effluent	
Suspended organic nitrogen	17.9
Soluble organic nitrogen	6.2
Ammonium	3.2
Nitrite + Nitrate	0.2
Remaining in water column	~58.0
Stored in sludge layer	~14.4
Ammonia volatilisation	0.0
Net recovery	~ 100

*The mass balance was calculated over a $3\times\theta_0$ period.

Overall these results indicate that the transformations of nitrogen fractions in M1 were mainly controlled by biochemical processes leading to ammonium removal by algal uptake, rather than any net nitrogen removal pathway. The differences in M1 pond performance in 2005 and 2006 were most likely due to photosynthetic activity variations associated with loading changes, as shown by the chlorophyll *a* values given in Tables 8.1 and 8.3. Therefore, ammonium nitrogen removal in a maturation pond is highly dependent on primary productivity.

8.3 Tracer Experiment with ^{15}N Stable Isotopes in Winter

Two tracer runs were carried out with ^{15}N stable isotopes in winter 2006/2007. M1 was firstly spiked with $^{15}NH_4Cl$ and then after $3\times\theta_0$ it was spiked again with $Na^{15}NO_2$. ^{15}N-labelled algae were not used in this experiment and ^{15}N-labelled nitrite was chosen as a tracer instead, considering that the in-pond algal biomass (organic nitrogen) is very low when the water temperature drops in winter. Moreover, it will help to elucidate the fate of oxidised forms of nitrogen (nitrite and nitrate) in WSP under winter conditions. Data from winter experiments (Figure 8.3) were collected for $6\times\theta_0$ in total; hence, $\delta^{15}N$ values from 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 spiked.

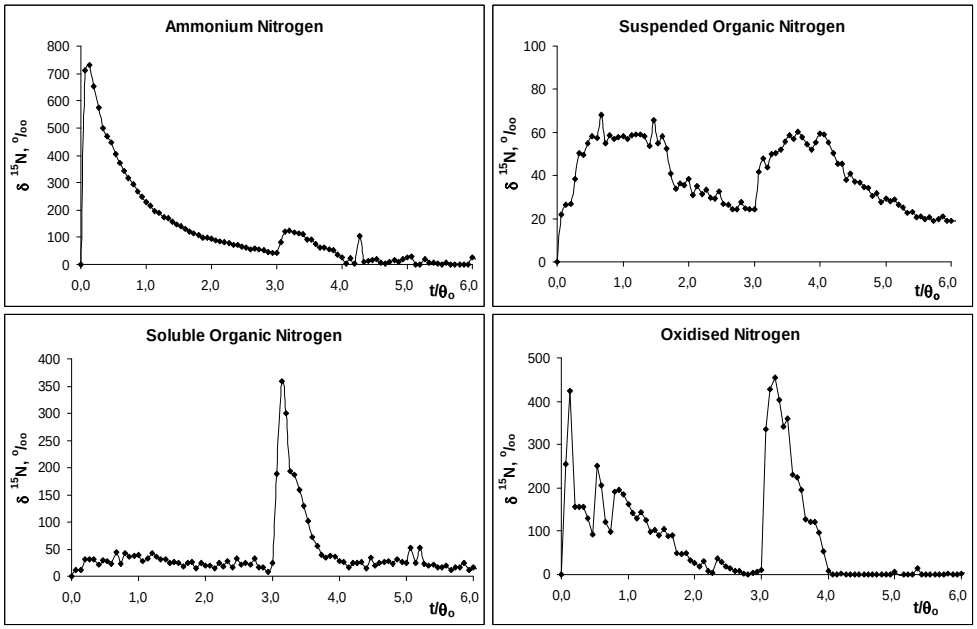


Figure 8.3 $\delta^{15}N$ values in nitrogen fraction from M1 effluent in winter 2006/2007

8.3.1 Tracer run with ^{15}N-labelled ammonium

Labelled ammonium decayed more slowly in winter (Figure 8.3) than during the corresponding tracer experiment in summer 2005 and it seems that ammonium oxidation was the preferred transformation pathway, followed by biological uptake. During the first half of the experimental timeframe ($0< t/\theta_0< 3$), all 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‰. 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 8.5). The ^{15}N mass balance showed also that ~10.0% of the tracer could not be accounted in any of the nitrogen fractions in M1 effluent or remaining inside M1 pond.

Table 8.5 Cumulative* ^{15}N-labelled ammonium recovery in winter 2006/2007

Nitrogen Fraction	Recovery, %
Recovered in M1 effluent	
Suspended organic nitrogen	2.0
Soluble organic nitrogen	0.8
Ammonium	55.7
Nitrite + Nitrate	3.0
Remaining in water column	~4.0
Stored in sludge layer	~24.5
Ammonia volatilisation	0.0
Net recovery	~90

* The mass balance was calculated over a $3\times\theta_0$ period ($0 < t/\theta_0< 3$)

Performance indicators from the weekly sampling (Table 8.6) showed that M1 received loadings of 6.2 kg BOD/ha d and 3.0 kg N/ha d. M1 was spiked with labelled ammonium during the coldest period in winter 2006-2007, when 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. Despite these seemingly adverse conditions, total nitrogen removal was removed at 813 g N/ha d (27%), which is surprisingly higher than in both the summer periods studied. However, 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).

Table 8.6 Water quality in M1 pond during experiments with $^{15}NH_4^+$ (winter 06/07)

Parameter	Influent	Effluent
SS, mg/l	24 ± 4	7 ± 1
BOD_5, mg O_2/l	23.7 ± 4.2	7.9 ± 0.9
Filtered BOD_5, mg O_2/l	5.7 ± 0.5	3.9 ± 0.4
TKN, mg N/l	8.4 ± 1.0	5.9 ± 0.2
Filtered TKN, mg N/l	5.2 ± 0.5	5.3 ± 0.3
NH_4^+, mg N/l	3.9 ± 0.5	4.7 ± 0.4
NO_3^-, mg N/l	0.46 ± 0.02	0.56 ± 0.03
NO_2^-, mg N/l	< 0.03	< 0.03
Chlorophyll *a*, μg/l	156 ± 22	34 ± 11
E. coli, $\log_{10}$ (cfu/100ml)	4	3

* average ± standard error

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 with a quarter of the total nitrogen removal rate (813 g N/ha d). Considering that (a) the oxidised nitrogen fraction in M1 effluent was highly enriched with ^{15}N tracer (~420‰), (b) ^{15}N tracer was not completely recovered (~90% recovery), (c) experiments to measure ammonia volatilisation rates reported a negligible contribution of that mechanism (<0.4–2.0 g N/ha d), but a clear enrichment of ex-pond gases with ^{15}N (16.17–35.77‰), and (e) there was no substantial nitrate accumulation in M1 effluent, it seams that total nitrogen removal in M1 pond could have been dominated by sedimentation of the incoming suspended organic nitrogen fraction and simultaneous nitrification-denitrification.

The nitrification-denitrification process has been considered as a minor nitrogen removal mechanism in WSP, basically because of low concentrations of nitrifying bacteria in the aerobic zone of the WSP, the lack of sufficient aerobic surface area for the attachment of the necessary nitrifying bacteria, the unlikely simultaneous presence of anaerobic and aerobic environments and low concentrations of nitrite and nitrate (Pearson, 2005; Reed, 1985; Ferrara and Avci, 1982). In contrast, Lai and Lam (1997) reported that nitrification-denitrification was the major nitrogen removal mechanism in a WSP in Melbourne, Australia, under cold weather conditions (autumn and early winter) when total nitrogen was more efficiently removed as compared with warm summer conditions.

The ^{15}N tracer experiments (bench-scale) carried out by Reddy (1983), under batch conditions, reported nitrogen losses in a control reactor with algal biomass for agricultural

drainage effluent treatment; in that particular case, when the system was spiked with ^{15}N-labelled nitrate, 28.9 percent of ^{15}N was unaccounted after a net mass balance. Reddy concluded that denitrification was probably the most important mechanism for permanent nitrogen removal. A net nitrogen removal via classical nitrification-denitrification in WSP would require the oxidation of ammonium to nitrate under aerobic conditions (e.g., in the pond water column), followed by the reduction of nitrate to molecular nitrogen under anoxic conditions (e.g., in the pond sediments); indeed WSP may develop such conditions thanks to the well-known diurnal variation of DO and redox potential, leading to oxidative conditions during the day and reductive conditions at night.

Considering that nitrogen algal uptake could reduce the availability of ammonia for nitrification, nitrification-denitrification may be the dominant mechanism for permanent nitrogen removal in WSP under conditions with low phytoplanktonic activity (e.g., late autumn and winter). In fact, high pH values in WSP reported under warmer weather conditions (pH >9 or even >10; Mara, 2004) may inhibit nitrification, as it is restrained at pH values above 8.5; nitrification is also inhibited when DO levels drop below 6 mg/l, whist nitrification rates are reduced (but not inhibited) at temperatures below 15ºC (Pearson, 2005).

During the tracer experiment with ^{15}N-labelled ammonium in winter, key parameters in M1 effluent such as pH, DO and ORP (redox potential) had a variation between 6.1–7.6, 5.4–9.0 mg/l and –46–311 mV, respectively; moreover, organic matter removal (BOD_5) in winter (67%) was higher than those reported in summer experiments (–43%, summer 2005; 5% summer 2006), suggesting an additional organic carbon demand (e.g., denitrification process) apart from that required for microbial respiration. Therefore, the alternation of oxidative and reductive conditions during day and night respectively, or the simultaneous presence of aerobic and anaerobic in-pond micro-ecosystems, may lead total nitrogen removal by nitrification-denitrification in shallow WSP (e.g., maturation ponds).

8.3.2 Tracer run with ^{15}N-labelled nitrite

M1 was spiked with $Na^{15}NO_2$ in order to elucidate the fate of oxidised forms of nitrogen (nitrite and nitrate) under winter conditions; the corresponding $\delta^{15}N$ values are shown in Figure 8.3 ($3< t/\theta_0< 6$). 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 (Table 8.7). M1 received loadings of 6.6 kg BOD/ha d and 1.8 kg N/ha d.

Table 8.7 Water quality in M1 pond during experiments with $^{15}NO_2^-$ (winter 2006/07)

Parameter	**Influent**	**Effluent**
SS, mg/l	15 ± 4	32 ± 4
BOD_5, mg O_2/l	23.3 ± 3.4	27.7 ± 4.3
Filtered BOD_5, mg O_2/l	10.2 ± 1.6	7.7 ± 1.4
TKN, mg N/l	6.5 ± 0.8	5.2 ± 0.5
Filtered TKN, mg N/l	4.9 ± 1.0	2.2 ± 0.4
NH_4^+, mg N/l	3.2 ± 0.9	0.8 ± 0.2
NO_3^-, mg N/l	0.32 ± 0.02	0.39 ± 0.02
NO_2^-, mg N/l	< 0.03	< 0.03
Chlorophyll *a*, μg/l	126 ± 48	226 ± 27
E. coli, $\log_{10}$ (cfu/100ml)	3	3

* average ± standard error

Labelled nitrite was rapidly transformed immediately after the tracer injection as it can be appreciated from the oxidised nitrogen fraction (Figure 8.3) which was enriched with ^{15}N up to 450‰ and decreased almost completely in only $1\times\theta_0$. Soluble organic nitrogen fraction was also highly enriched (~360‰), as well as ammonium nitrogen (~120‰) and suspended organic nitrogen (~60‰). Results from a ^{15}N mass balance for $3\times\theta_0$ ($3< t/\theta_0< 6$) showed that the labelled nitrite tracer was poorly recovered in the M1 effluent (suspended organic fraction, 3.3%; ammonium nitrogen fraction, 1.9%; soluble organic fraction, 4.8%; and nitrate fraction, 1.5%). 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 in any of those fractions.

Considering the ^{15}N enrichment (16.17–35.77‰) in samples collected from ammonia volatilisation experiments carried out during the previous ^{15}N-tracer spike in winter, it was decided to change the 2% boric acid solution in the ammonia absorption system for a 1% sodium hydroxide solution during the second tracer spike in winter with ^{15}N-labelled nitrite. It would increase the capacity of the system to absorb acid gases coming out from M1 pond, such as NOx. 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 (NOx) leaving M1 through pond surface area to the atmosphere.

The behaviour of ^{15}N-labelled nitrite indicates that it may be involved in different transformations occurring simultaneously such as (a) nitrite oxidation to nitrate (catalysed by the membrane-bound enzyme nitrite oxidoreductase); (b) nitrite reduction to N_2, including intermediate steps like NO formation (catalysed by two distinct types of enzymes: cytochrome *cd*$_1$ and copper-containing nitrite reductase; heme-*cd*$_1$-NiR and copper-NiR, respectively), N_2O formation (catalysed by three types of nitric oxide reductase (NOR) enzymes: cNOR, qNOR and qCuNOR), and final N_2 formation (catalysed by nitrous oxide reductase (NOS) enzyme); (c) anaerobic ammonium oxidation (catalysed by ANAMOX enzymes); and/or (d) nitrite reduction to ammonium for bacteria and algae uptake (catalysed by the cytochrome c nitrite reductase (NrfA) enzyme) (Kool *et al.*, 2007; Butler and Richardson, 2005).

This indicates that the transformations of ^{15}N-labelled nitrite nitrogen are intermediated by enzymes (proteins), which could have been extracted on the soluble organic fraction during sequential nitrogen partitioning (section 3.6.2) and therefore it may explain the considerable increment of ^{15}N in that nitrogen fraction (~360‰, δ^{15}N) immediately after the tracer was injected. Moreover, considering that about 43 percent of the tracer was not recovered, that ammonia volatilisation was also negligible during winter experiments (<0.4–2.0 g N/ha d) and that the presence of acid gases coming out M1 pond was detected, the unaccounted ^{15}N from the mass balance could have left M1 pond as a consequence of a denitrification process.

Tracer experiments carried out under winter conditions 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 algae growth (winter), ammonium nitrogen was mainly transformed into oxidised nitrogen (nitrite and nitrate) and then permanently removed via denitrification process (total nitrogen removal = 27%; ammonium removal = 0%). On the other hand, when environmental conditions were more favourable for phytoplanktonic activity (late winter – early spring), ammonium nitrogen was removed more efficiently (75%) by algal uptake, increasing the suspended nitrogen fraction in M1 effluent by 88 percent, and simultaneously removed by nitrification-denitrification (total nitrogen removal = 18%).

8.4 Results for Molecular Microbiology Analyses

Molecular microbiology analyses reported the presence of specialised microorganisms belonging to both oxidation and reduction parts of the nitrogen cycle (Table 8.8). Ammonia-oxidising bacteria (AOB) were identified as uncultured *Nitrosomonas* sp AF527015 (Rowan, 2003) confirming that the maturation WSP under study has the availability to perform ammonia oxidation to nitrite and possibly, denitrification in anoxic and low-oxygen environments (e.g., winter conditions) (Schmidt *et al*., 2002, 2003); Ammonia-oxidising archaea (AOA) were not found in any of the samples processed.

Results of PCR amplification targeting nitrite-oxidising bacteria (NOB) were positive for all samples analysed, revealing the presence of nitrifiers in M1 pond. Unfortunately, although the DGGE gels appeared to confirm the presence of NOB, *Nitrobacter* sp. or *Nitrospira* sp. were not detected when the bands were sequenced in the BLAST[6] search. That may suggest that NOB are not playing a major role or at the very least a small one.

Table 8.8 Results for molecular microbiology analyses (presence/absence)

Sample	Ammonia-oxidisers		Anammox	Methanotrophs		Denitrifiers		Nitrifiers
	AOB	**AOA**	**Pla46 and Amx368**	**pmo682**	**MB661**	***nirS***	***nirK***	
Summer 2006								
Water column								
10cm depth	+	–	–	–	–	+	+	+
45cm depth	+	–	–	–	–	+	+	+
80cm depth	+	–	–	–	–	+	+	+
Effluent	+	–	–	–	–	+	+	+
Pond's wall	+	–	–	–	–	+	++	+
Sludge	+	–	–	–	–	+	+	+
Winter 2006/2007								
Water column								
Composite	+	±±±	–	++	+	+	+	+
Sludge	+	–	–	+	+	+	+	+

Key: ++ = strong band present; + = band present; ±±±; extremely weak band; – = no band present.

On the other hand, methanotrophs (e.g., *Methylocystis* sp., *Methylosinus* sp.) were present only in samples collected during winter experiments, when environment conditions could have been oxygen-limiting for longer. Considering that the co-methabolism of ammonium by methane-oxidising bacteria as methane mono-oxygenase is very similar to

[6] BLAST: Basic Local Alignment Search Tool is an algorithm for comparing primary biological sequence information, such as the amino-acid sequences of different proteins or the nucleotides of DNA sequences.

ammonium mono-oxygenase, methanotrophs can also catalyze the oxidation of ammonium (nitrification) and produce nitric and nitrous oxides (Murrell and Radajewski, 2000). Methanotrophs are not themselves known to carry out denitrification, however, 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., NOx, N_2) and so contributes to permanent nitrogen removal in WSP.

The reduction of nitrite to nitric oxide distinguishes denitrifiers from other nitrate-respiring bacteria and that reaction is catalysed by two different types of nitrite reductases (Nir) either a cytochrome cd_1 enzyme or a Cu-containing enzyme, encoded by the functional genes *nirS* and *nirK*, respectively (Throbäck *et al*., 2004). Results of PCR amplification targeting *nirS* and *nirK* genes revealed the presence of denitrifiers in M1 pond, not only in samples collected from the pond sidewall and sludge layer as expected, but also in water column samples. Therefore, denitrification supported either by nitrifiers or methanotrophs in WSP may be counted as a feasible mechanism for permanent nitrogen removal both in summer and winter, but its relative supremacy over other nitrogen removal mechanisms (e.g., biological uptake) would depend upon algal activity.

Although nitrification-denitrification has been reported as the main mechanism for permanent nitrogen removal in WSP by others researchers (e.g., Somiya and Fujii, 1984; Lai and Lam, 1997; Zimmo *et al*., 2003; Picot *et al*., 2005; Strang and Wareham, 2005; Picot *et al*., 2007), they did not provide any evidence related to nitrogen transformation pathways dominating nitrification and denitrification, apart from Picot *et al*. (2007) who detected N_2O gases in samples collected from maturation ponds in France. The results reported in this research work including ^{15}N tracer experiments and molecular microbiology analysis are new evidence to support that nitrification-denitrification is one of the major mechanisms for permanent nitrogen removal in WSP.

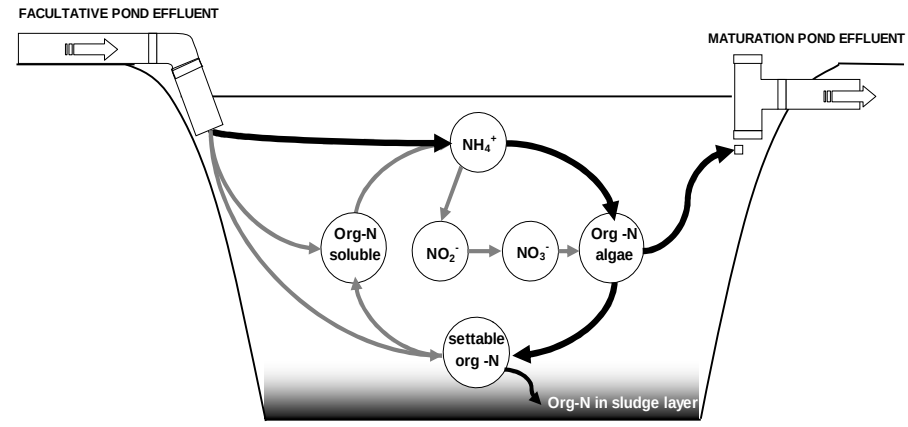
Finally, the possibility of anaerobic ammonium oxidation in M1 pond was discounted as a feasible nitrogen removal mechanism because there was no presence of any targeted groups (*Planctomycetales* and Anammox bacteria). Therefore, nitrogen losses via the ANNAMOX process were not performed in M1.

8.5 Descriptive Model for Nitrogen Transformations and Removal in WSP

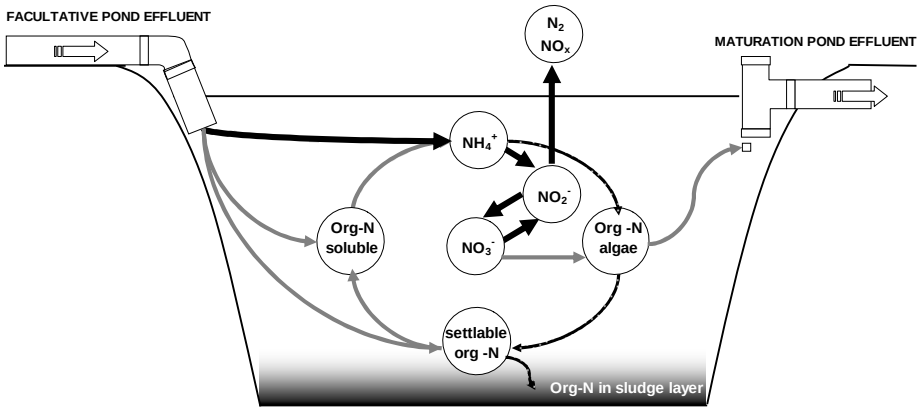
Stable isotope analysis of $\delta^{15}N$ showed that the nitrogen cycle in maturation ponds is dominated in summer by biological uptake as ammonium nitrogen is rapidly transformed

into algal biomass as suspended organic nitrogen which then either leaves in the pond effluent or is sedimented as dead cells. Ammonia removal by volatilisation makes little or no contribution to nitrogen removal either in summer or winter; moreover, nitrification is masked by biological nitrate uptake in summer, under completely aerobic conditions (day and night), and simultaneous denitrification in winter.

Thus algal uptake of ammonium and subsequent sedimentation and retention in the sludge layer, after partial ammonification of the algal organic nitrogen, appears to be the dominant mechanism for permanent nitrogen removal in maturation ponds during warm summer months when pH and temperatures reach their annual maxima. In contrast, ammonium oxidation by ammonia oxidising bacteria, methanotrophs and/or nitrifiers leading to partial or total nitrification, and simultaneous denitrification may be the dominant mechanism of total nitrogen removal during the cold winter months in temperate climates. A descriptive model is presented in Figure 8.4.



(a) Under favourable conditions for algal growth



(b) Under unfavourable conditions for algal growth

Figure 8.4 Descriptive model for nitrogen transformation and removal in WSP

8.6 Related Publications

This research work was partially published as part of the conference proceedings of five national and international conferences, including the 7th IWA Specialist Conference on Waste Stabilization Ponds held at the Asian Institute of Technology in Bangkok, 25–27 September 2006; the corresponding paper was selected for publication in *Water Science and Technology*. Related publications are listed as follows:

Camargo Valero, M. A. and Mara D. D. (2008). Revealing the dynamics of nitrogen transformations and removal mechanisms in maturation waste stabilization ponds by tracer experiments with ^{15}N stable isotopes. *In* Proceedings of the 4th IWA International Young Water Professionals Conference, University of California, Berkeley, USA, 16–18 July.

Camargo Valero, M. A. and Mara D. D. (2008). The dynamics of nitrogen cycle in domestic wastewater treatment by waste stabilisation ponds (in Spanish). *In* Proceedings of the 1st Colombian Congress of Microbiology, Universidad de Antioquia, Medellín, Colombia, 1–3 May.

Camargo Valero, M. A. and Mara D. D. (2007). Nitrogen transformation and removal in maturation ponds: tracer experiments with ^{15}N stable isotopes in the United Kingdom in summer. *In* Proceedings of the II International Conference SmallWat07, Seville, 11−15 November.

Camargo Valero, M. A. and Mara D. D. (2007). Nitrogen transformation pathways and removal mechanisms in maturation ponds in the UK in summer. *In* Proceedings of the 8th UK Meeting - IWA UK Young Water Professionals Conference, University of Surrey, Guildford, 18–20 April.

Camargo Valero, M. A. and Mara D. D. (2007). Nitrogen removal in maturation ponds: tracer experiments with ^{15}N-labelled ammonia. *Water Science and Technology*, **55**(11), 81-85.