3. MATERIALS AND METHODS

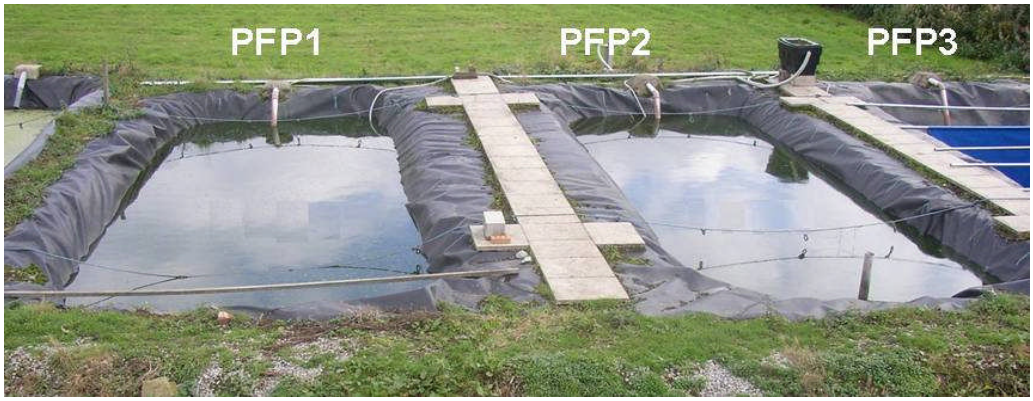
This chapter describes the materials and methods used in the study of nitrogen transformation pathways and removal mechanisms in two pilot-scale maturation ponds located at Esholt WWTP, Bradford, West Yorkshire, UK. A description of the pilot-scale WSP system at Esholt is given, followed by the hydraulic and physical characterization of the two maturation ponds under study and the performance monitoring programme undertaken, which includes both water quality and flow rate surveys. Moreover, a detailed description of experiments carried out to assess the importance of specific nitrogen removal mechanisms such as ammonia volatilisation and sedimentation of organic nitrogen via biological uptake, is reported herein. Finally, tracer experiments with ^{15}N stable isotopes under summer and winter conditions are detailed, as well as complementary analysis based on molecular microbiology techniques.

3.1 PILOT-SCALE WSP SYSTEM

The University of Leeds pilot-scale Waste Stabilisation Pond (WSP) experimental facilities are located at Yorkshire Water's Esholt Wastewater Treatment Works in Bradford, West Yorkshire, UK (53°50'54"N, 1°42'42"W). The Esholt works serve a population equivalent of 623,696 inhabitants, of which 49 percent is trade waste mainly from chemical manufacturing and dyeing industries (Abis, 2002). The pilot-scale WSP system comprises primary treatment by three primary facultative ponds in parallel (PFP1, PFP2 and PFP3), as described by Abis (2002); each PFP is receiving wastewater from the main screened sewage line which feeds Esholt WWTP. Secondary treatment is carried out in either two maturation ponds (M1 and M2) and a reed-bed channel (RBC) in series, or three horizontal-flow rock filters in parallel including one working as a control filter (CRF), one as a subsurface horizontal-flow constructed wetland (CW), and one as aerated rock filter (ARF) (Figure 3.1).

The treatment train used in this research comprises one primary facultative pond (PFP1), two maturation ponds in series (M1 and M2) and a reedbed channel (RBC). However, this research project is focussed on the performance of the M1 and M2 ponds with regard to nitrogen transformations and removal mechanisms exclusively. PFP1 was fed with screened wastewater at average loading rates of 80 kg BOD_5/ha d and 8 kg N/ha d, with an average nominal retention time (θ_0) of 60 days. These operational conditions for

primary facultative ponds were found to be optimal by Abis (2002) for typical weather conditions in the UK.



(a) Primary facultative ponds

(b) Maturation ponds and reed bed channel

(c) Rock filters

Figure 3.1 Pilot-scale WSP system at Esholt, Bradford.

The maturation ponds were constructed in June 2004 and their design was based on prior results reported by Johnson and Mara (2002). Each maturation pond is 6.25 × 3.45 × 1.00 m and they were lined with a 1.0 mm high-density polyethylene (HDPE) membrane. Two peristaltic pumps (model 504S, Watson Marlow Bredel Inc., Wilmington, USA) in parallel were used to pump the effluent from PFP1 to the first maturation pond (M1), while the second maturation pond (M2) was fed with the effluent of M1 by gravity via a PVC pipe (110 mm) fitted with a T-piece. The inlet and outlet structures were arranged at diagonally opposite corners to improve mixing and to reduce short-circuiting and dead spaces.

The M2 effluent was discharged into a reedbed channel (10 × 0.5 × 0.5 m) which was lined with a 1.0 mm HDPE liner and a 75-mm layer of river sand placed on the bottom of the channel to protect the liner. The bed was filled with clean limestone aggregate: 40–

100 mm for the first two metres of the channel, followed by 20 mm for the rest of the channel; it was mainly planted with *Typha, Glyceria* and *Iris* in a random order along the length of the bed (Johnson and Mara, 2002). RBC effluent was returned to the screened sewage line.

3.2 PHYSICAL AND HYDRAULIC CHARACTERIZATION

3.2.1 Physical Characterization

M1 and M2 maturation ponds were physically characterized in order to determine net pond volume (*V*); hence, a bathimetry survey was carried out on site. Pond depth readings were taken every 50 cm along the pond length and width. Collected data were processed in order to determine the pond volume and other key parameters such as average depth and surface area. The white towel test (Malan, 1964) was carried out simultaneously to determine the depth of the sludge layer in each pond.

3.2.2 Hydraulic Characterization

Flow rates and theoretical retention times

The primary facultative pond effluent coming into M1 was measured weekly following a volumetric method (readings from a stopwatch and a measuring cylinder were taken). M1 and M2 effluents were calculated from a water flow balance (equation 3.1); absence of infiltration through pond bottoms was assumed, considering that the pond lining was impermeable. The net evaporation rate (rainfall rate minus evaporation rate) was estimated from weekly readings using a hook gauge evaporimeter (Casella CEL Ltd., Bedford, England) located on site, next to the primary facultative ponds.

$$Qe = Qo + (1{,}000 \times E_{net} \times A) \qquad \textbf{(3.1)}$$

where *Qo* and *Qe* are inlet and outlet pond flow rates (m^3/d), respectively; E_{net} is the net evaporation rate (mm/d) and *A* is the pond's surface area (m^2). Theoretical hydraulic retention time (θ_0) for M1 and M2 maturation ponds were also calculated considering inlet and outlet flow rates and net pond volume (*V*, m^3) (equation 3.2).

$$\theta_0 = \frac{V}{(Qo + Qe)/2} \qquad \textbf{(3.2)}$$

Hydraulic flow regime

Tracer experiments with Rhodamine WT (20% w/v) were conducted in order to determine the pond hydraulic mixing characteristics. Hence, M1 was spiked in three separate runs with 50 ml of a solution containing a known amount of tracer. The first run was undertaken on 19 July 2005 with 4.0793 g of Rhodamine WT; the corresponding dates and mass of Rhodamine WT for the second and third runs were: 22 June 2006, 4.0873g; and 20 December 2006, 2.3001g. Tracer concentrations in the M1 effluent were measured in-situ, every 20 minutes for $1\times\theta_0$ before spiking and for $3\times\theta_0$ afterwards, with a Rhodamine WT fluorometric sensor (model YSI 6130, YSI Inc., Yellow Springs, USA) coupled to a multiparameter sonde (YSI 6820; YSI Inc.) with continuous data-logging system. Data from Rhodamine WT tracer experiments were also processed following the method described by Levenspiel (1999) for dispersion number, actual retention time and tracer recovery; the dead-space and short-circuiting indices were also calculated by the method given by Kilani and Ogunronbi (1984).

3.3 PERFORMANCE MONITORING PROGRAMME

The performance of the two maturation ponds in series was monitored in order to determine the seasonal pattern of nitrogen compounds transformations and removal. M1 and M2 maturation ponds were monitored on a weekly basis from October 2004 to May 2007, by means of grab samples collected in the mornings (10 am - noon) from influent and effluent of each treatment unit and pond water column as described by Pearson *et al.* (1987b). Sampling points were named as illustrated in the figure 3.2.

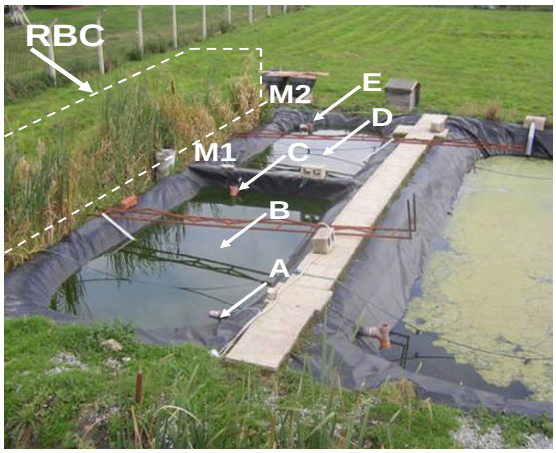


Figure 3.2 Sampling points for wastewater quality surveys

Collected water samples were taken to the Public Health Laboratory, University of Leeds, and processed the same day as detailed in Table 3.1. Samples A, B and D were also examined weekly for algal diversity by using an upright microscope (model BHX-2, Olympus, Melville, NY, USA). Moreover, filtered samples from A, C and E sampling points were analyzed biweekly for sulphate, phosphate, nitrite and nitrate by ion chromatography (IC-ED; DX500, Dionex Cop., Sunnyvale, CA, USA). Microscopic examination and ion chromatography work on weekly samples was carried out by Mrs Karen Stevens (School of Civil Engineering, University of Leeds) on behalf of the author.

Table 3.1 List of laboratory analysis conducted in water samples from M1 and M2

Parameter	**Method***	**Sample****									
		A	**A+**	**B**	**B+**	**C**	**C+**	**D**	**D+**	**E**	**E+**
Ammonium	4500-NH_3 B	✓		✓		✓		✓		✓	
Alkalinity	2320 A		✓		✓		✓		✓		✓
Bacteriochlorophyll	Pearson *et al*. (1987a)	✓		✓		✓		✓		✓	
BOD_5	5210 B	✓	✓			✓	✓			✓	✓
Chlorophyll-*a*	Pearson *et al*. (1987a)	✓		✓		✓		✓		✓	
COD	5220 C	✓	✓			✓	✓			✓	✓
Faecal coliforms	9222 D	✓				✓				✓	
Suspended solids	2540 D	✓		✓		✓		✓		✓	
TKN	4500-N_{org} C	✓	✓			✓	✓			✓	✓
Volatile suspended solids	2540 E	✓		✓		✓		✓		✓	

* Standard Methods for Examination of Water and Wastewater (APHA, 1998), unless otherwise stated
** Water sample: A = M1 influent, B = M1 column, C = M1 effluent, D = M2 column, E = M2 effluent
+ Filtered sample

Simultaneously, a multiparameter sonde (YSI 6820; YSI Inc.) was used on a weekly basis to measure on site dissolved oxygen (DO), conductivity, oxidation-reduction potential (ORP), temperature and pH from pond influent and effluent and pond water column at 0.10, 0.45 and 0.80 m depth. Moreover, water column temperature in M1 and M2 were monitored on an hourly basis at 0.10 and 0.80 m depth using a digital temperature data logger (Thermochron iButtons, Maxim Integrated Products, Inc., Sunnyvale, CA, USA). Climatic conditions at Esholt were also estimated from data provided by the Met Office UK from a weather station located in Bradford, West Yorkshire (Location 4149E 4352N).

3.4 NITROGEN REMOVAL BY AMMONIA VOLATILISATION

Ammonia volatilisation is one of the feasible nitrogen removal mechanisms in WSP. It has generally been reported as (or assumed to be) the main nitrogen removal mechanism in WSP; this hypothesis is based on two observations: (a) in-pond pH values can reach high values (>9, even >10), so increasing the proportion of the total ammonia present as the un-ionized form (NH_3), and (b) in-pond temperatures can also be high, so improving the mass transfer rate of NH_3 to the atmosphere. However, the importance of ammonia volatilisation on nitrogen control in domestic wastewater treatment by WSP has been based on theoretical analysis. Models used to predict ammonia volatilisation from WSP (e.g., Pano and Middlebrooks, 1982; Rockne and Brezonik, 2006) have not been calibrated nor validated by means of direct measurements of volatilized ammonia.

An apparatus was designed to collect ammonia gas coming out from WSP. The apparatus has a capture chamber and an absorption system, which were optimized under laboratory conditions prior to being used to assess ammonia volatilisation rates in our pilot-scale WSP system at Esholt. That design was based on previous works carried out by Shilton (1996), Zimmo *et al*. (2003), Epworth (2004) and Caicedo Bejarano (2005).

3.4.1 Laboratory Experiments

Ammonia losses from water enriched with ammonium chloride were assessed under controlled conditions in the laboratory to estimate the recovery percentage achieved by a simple apparatus designed to collect and absorb ammonia; that apparatus has a capture chamber and an absorption system. Three absorption systems and two capture chambers were tested in triplicate in the laboratory to estimate ammonia volatilisation rates. The three absorption systems (AS1, AS2 and AS3) were tested using a controlled capture system which comprised a 25-l container with a lid with an air inlet and outlet on opposite sides (Figure 3.3).

Absorption system AS1 comprised two 250-ml conical flasks; AS2, three 250-ml conical flasks; and AS3, a packed column (height 40 cm; diameter 100 mm) and three 250-ml conical flasks. The packed column had 20-mm PVC spheres at the base, silicon rings (length, 15 mm; diameter, 5 mm) in the middle, and glass rings (length, 8 mm; diameter, 6 mm) at the top. The packed column and conical flasks were filled with 1,500 and 200 ml of a 2% boric acid solution, respectively, and a 25-mm sandstone was placed on the bottom of each as an air diffuser.

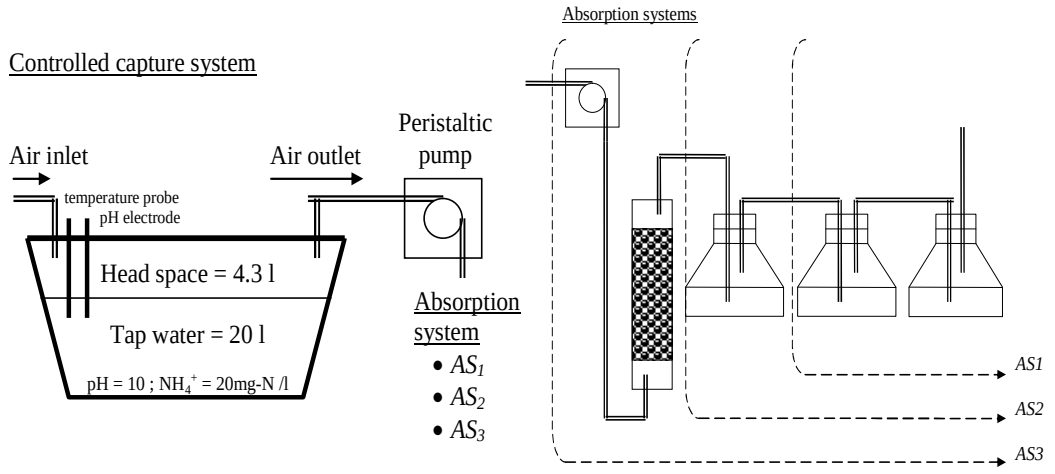


Figure 3.3 Experimental set-up for selecting an ammonia absorption system

The water sample in the capture chamber was 20 l of tap water enriched with NH_4Cl to a concentration of 20 mg N/l; the pH was adjusted to 10 with 2N NaOH. A constant air flow of 2.6 l/min was drawn through the head space above the water surface using a peristaltic pump (model 624S, Watson Marlow Bredel Inc.). The pump outlet was connected to the absorption system, forcing the head-space gases to bubble-up through a 2% boric acid solution as mentioned before, so absorbing the volatilized ammonia. Water samples were collected from the container at the beginning of the experiment and then after 24 hours; water and boric acid samples were processed for NH_4^+ (method 4500-NH3 B; APHA, 1998). Temperature and pH were continuously monitored with a Corning® model 240 pH meter. Each absorption system was tested in triplicate and then the best absorption system was chosen based on the criterion of maximal ammonia recovery.

Two capture chambers were tested on a 150-litre container which was filled with 120 l of ammonium enriched water as described above (Figure 3.4). The first capture chamber (CC1) was a perspex box (0.34 × 0.33 × 0.50 m) with an air inlet and outlet on opposite sides; and the second one (CC2) was the same perspex box but with three equally spaced plastic baffles. A constant air flow of 2.6 l/min was drawn through the head space above the water surface in the capture chamber, using a peristaltic pump (model 624S, Watson Marlow Bredel Inc.), and then into the AS3 absorption system.

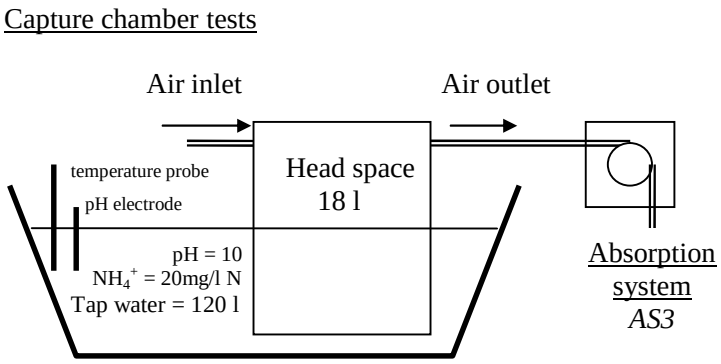


Figure 3.4 Experimental set-up for capture chamber testing

Temperature and pH were continuously monitored by using a multiparameter sonde (YSI 6820; YSI Inc.), and water samples were collected from the container at the beginning of the experiment and then after 24 hours; water and boric acid samples were processed for NH_4^+ as described previously. Each capture chamber was tested in triplicate and the better one chosen based on maximal ammonia recovery; the airflow was then optimized to improve ammonia recovery.

3.4.2 On-site Experiments

Laboratory results were analysed to choose the best capture chamber, absorption system and air flow rate which were used in further experiments on our pilot-scale WSP system. On-site experiments were carried out in summer 2005, under favourable conditions for ammonia volatilisation (e.g., high in-pond temperature and pH) in the first pond (M1) of the two existing maturation ponds in series. Ammonia volatilisation rates from M1 were measured over 72-h periods. Boric acid solution samples from the absorption system were collected and processed for NH_4^+ and a blank control was used to account for ammonia interference from the surrounding air on site. A complementary set of experiments was also carried out under winter conditions (winter 2006/2007).

A net nitrogen mass balance over the experimental timeframe was calculated based on results from the hydraulic characterization (section 3.2.2), performance monitoring programme (section 3.3) and nitrogen sedimentation rates (section 3.5). Temperature, DO and pH from M1 effluent were also measured on site in real time using a multiparameter sonde (YSI 6820; YSI Inc.).

3.5 SEDIMENTATION OF ORGANIC NITROGEN VIA BIOLOGICAL UPTAKE

This section describes an experiment carried out to estimate a net nitrogen accumulation rate in sediments from maturation ponds and to determine the relative importance of the sedimentation of organic nitrogen via biological uptake (dead algae and heterotrophic bacteria) within the mechanisms involved in nitrogen removal in WSP.

Settled organic nitrogen samples from M1 and M2 maturation ponds were collected seasonally in 10-litre metal buckets which were strategically placed on the bottom of each pond (two buckets per pond) and taken out at the end of each season. Collected sediment samples were sieved (ASTM sieve No. 10) to remove coarse solids and settled in 1-litre Imhoff cones. Thickened sediment samples were processed for solids and moisture content (methods 2540 F and 2540 G; APHA, 1998).

Additional sludge samples were collected on a monthly basis from the bottom of M1 and M2 and processed as described previously. Thickened sub-samples (seasonally and monthly sampling) were dried at 105ºC and processed simultaneously for nitrogen content (dry base) and ^{15}N:^{14}N ratios (section 3.6) using an elemental analyzer coupled with a stable isotope ratio mass spectrophotometer (EA-IRMS; EuroEA3000-Micromass Isoprime, Eurovector, Milan).

Nitrogen sedimentation rates (N_{sed}, g N/ha d) were calculated by using the following equation:

$$N_{sed} = 10{,}000 \times \left(\frac{V_{TS} \times TS \times N}{A \times t} \right) \qquad \textbf{(3.4)}$$

where V_{TS} and TS are the volume (l) and the content of total solids (g/l) from thickened sediment samples, respectively; N is the nitrogen content (fraction) in dry sediment samples, A is the sum of the top area of each bucket (m^2) and t is the time of sampling (d).

3.6 TRACER EXPERIMENTS WITH ^{15}N STABLE ISOTOPES

Tracer experiments using ^{15}N stable isotopes were carried out in our pilot-scale WSP to facilitate the study of processes involving both inorganic and organic forms of nitrogen, in order to determine the relative importance of nitrogen transformations and removal associated with ammonia volatilisation, nitrification, denitrification, and algal uptake and its subsequent sedimentation and retention/hydrolysis in the sludge layer. In some cases, these feasible nitrogen removal mechanisms may proceed simultaneously and only by

tracking labelled nitrogen species, it may be possible to distinguish each process contribution. Therefore, M1 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*).

3.6.1 ^{15}N Spiking and Sampling

The M1 maturation pond was spiked to increase the $\delta^{15}N$ of dissolved ammonium effluent by 1000‰ with a single pulse of 0.6812 g of $^{15}NH_4Cl$ in summer 2005 and with 1.2516 g (dry solids) of ^{15}N-labelled algae in summer 2006. The ^{15}N-labelled algae were produced by culturing *Chlorella vulgaris* (CCAP 211/11B; SAMS Research Services Ltd, Oban, Scotland) in 10 litres of Bold's basal medium (Andersen *et al*., 2005), substituting the nitrogen source with $^{15}NH_4Cl$. M1 was also spiked twice in winter 2006-2007: firstly with $^{15}NH_4Cl$ (0.6822 g) and, after $3\times\theta_0$, with $Na^{15}NO_2$ (1.0 g). ^{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 each spiking and for $3\times\theta_0$ 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 (YSI 6820; YSI Inc.) was used to measure in real time DO, temperature and pH in the M1 effluent.

3.6.2 Laboratory Analyses

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).

Composite samples were also sequentially partitioned to extract four nitrogen species separately (Figure 3.5): (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 volatilisation 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).

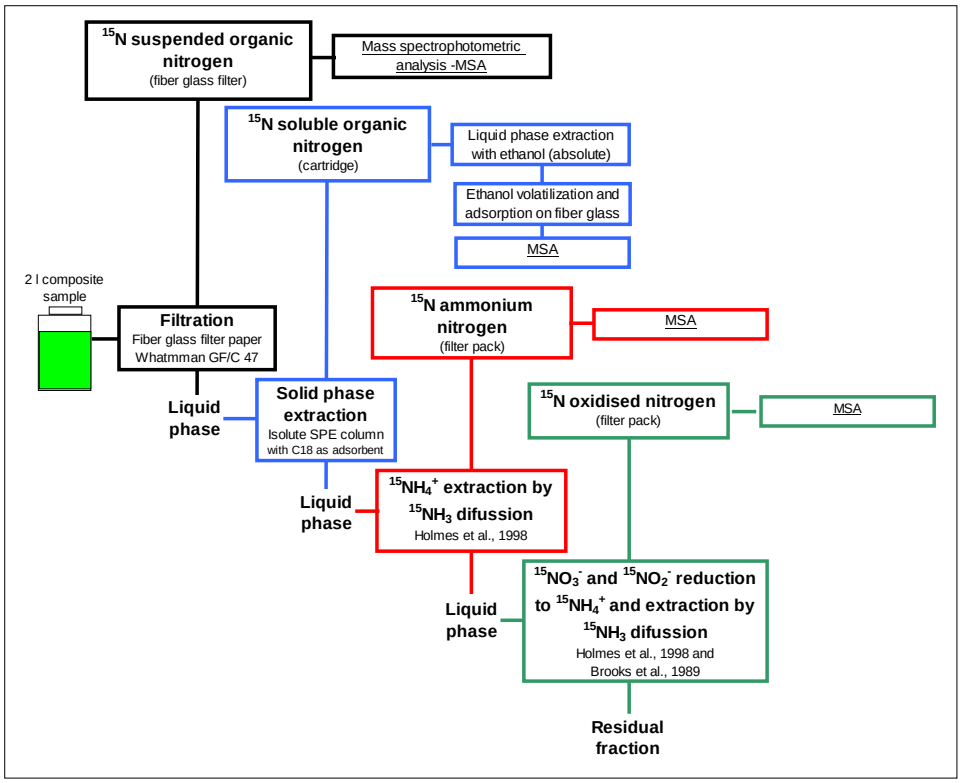


Figure 3.5 Physical and chemical sequential extraction for nitrogen partitioning

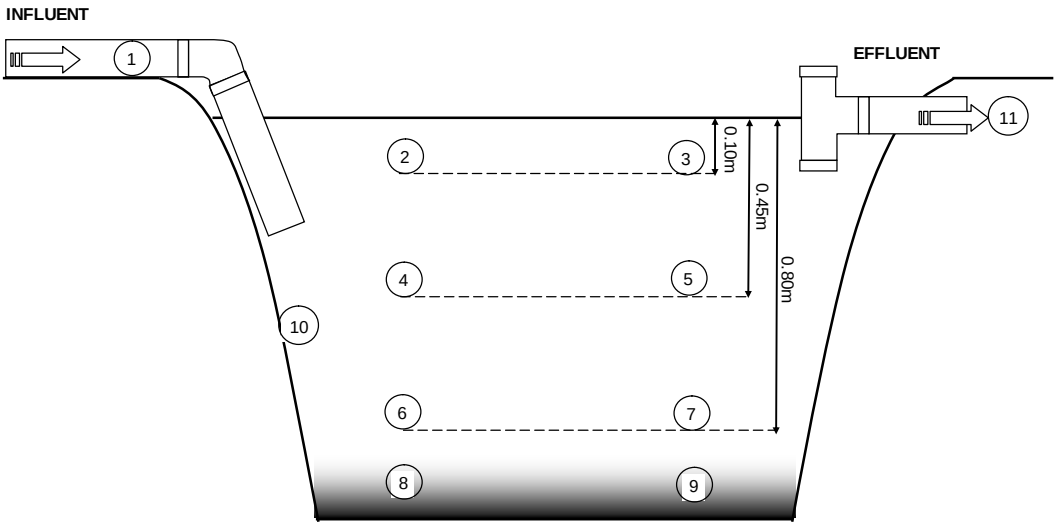
Labelled ammonia losses by volatilisation were estimated on site following the optimized procedure as illustrated in section 3.4.1; boric acid samples from the ammonia absorption system were processed for ammonium and ^{15}N:^{14}N ratios, as described previously. Settled organic ^{15}N was estimated from sediment samples, which were collected and processed as described in section 3.5. Additionally, data for nitrogen transformation and removal performance indicators (e.g., TKN, NH_4^+, NO_2^-, NO_3^-) from the M1 influent, water column and effluent were obtained from the performance monitoring programme (section 3.3).

3.7 MOLECULAR MICROBIOLOGY ANALYSES

The molecular microbiology work was undertaken to identify the presence of specialised groups of microorganisms belonging to the nitrogen cycle in wastewater treatment. Target groups were ammonia oxidisers (bacteria and archea), anaerobic ammonia oxidisers (anammox), methane-oxidising bacteria (methanotrophs), nitrifiers and denitrifiers.

3.7.1 Sampling and Sample Preservation

Water and sludge samples were collected from M1 and M2 on a monthly basis for molecular microbiology analyses. Sampling points were selected in order to obtain representative samples from pond environment (water column, pond's walls and sludge layer) as illustrated in Figure 3.6. Collected samples were preserved with absolute ethanol (1:1 v/v) and stored at −20ºC in the laboratory before being processed.



Samples for molecular microbiology analyses: Influent (1); water column at 0.10m (2 and 3), 0.45m (4 and 5) and 0,80m depth (6 and 7); sludge layer (8 and 9); pond's wall (10) and effluent (11).

Figure 3.6 Sampling points for molecular microbiology analyses

3.7.2 Laboratory Analyses

The molecular microbiology work was carried out in the School of Civil Engineering and Geosciences, University of Newcastle Upon Tyne, by Mrs Fiona Read and Dr Russell Davenport on behalf of the author. 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 3.2). 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.

Table 3.2 List of 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
GGAGRAAAGYAGGGGATCG CTAGCYTTGTAGTTTCAAACGC	CTO189f-CTO654r	Bacterial *amoA*, ammonia mono-oxygenase	ß-proteobacterial ammonia oxidizing bacteria (AOB) (Kowalchuk *et al.*, 1997; 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
AGAGTTTGATCMTGGCTCAG CACCTGTGCTCCATGCTCCG	Bact27f-Nbac1050r	16S ribosomal RNA	*Nitrobacter* nitrite-oxidizing bacteria (Freitag *et al.*, (2005)
GTSAACGTSAAGGARACSGGGA STTCGGRTGSGTC 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

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 3.2), 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 15N tracer experiment results.