Chapter 4 ~ Research Materials and Methods

4.1 Introduction

This chapter presents all aspects of the research and experimental methodology, conducted both in the laboratory and on site at Esholt, over the three-year research period. Site procedures and operational intricacies are also recorded in detail. Site-based experimental methods are documented, and individual experiments at a micro-scale described. Laboratory methods employed to process samples, and a sample processing format are referenced and catalogued in full.

In essence, the two pilot-scale primary facultative ponds (PFP's) used for the experimental period were analysed and monitored weekly over a two and a half year period. One main portion of the data represents this weekly sampling regime – the analytical methods used for all these samples are given in section 4.4.1. Weekly analysis was critically important, not only in order to assess operational and maintenance procedures responsible for healthy pond performance, and to highlight any problems that were occurring, but also to contextualise the data provided by other experiments carried out in parallel. Individual studies which ran in parallel to the weekly sampling strategy are described herein. These are the assessment of ammonia volatilization (section 4.6), molecular microbiological analysis (4.7) and sludge accumulation and analysis (4.8).

The major portion of the experimental work was given over to the stable isotope tracer experiments, the methodology of which described in section 4.5. The isotopically stable compound of labelled ammonium chloride ($^{15}NH_4Cl$) containing a Rhodamine WT slug, was prepared and introduced to the PFP as a liquid spike. This simultaneously enabled the ^{15}N to be traced throughout the system, and the hydraulic conditions of each experimental run to be determined. Each spike was introduced as a single pulse into the influent flow. The $^{15}NH_4Cl$ underwent biochemical transformations within the pond, and samples were taken every hour throughout each experimental run, to capture these transformations at the pond effluent. Rhodamine WT is a reliable dye tracer which produced a pulse, or series of pulses, as it passed out of the pond at the effluent point. The capture and analysis of these data enabled the hydraulic characteristics of the pond to be

mapped, and a dispersion number (D/uL) obtained to describe the mixing regime in that pond. Site-based methods are detailed here, as are the partitioning of samples in the laboratory, to extract the different ^{15}N fractions bound in each sample.

4.2 The experimental WSP systems: site-based methods

Two of the three experimental pilot-scale PFP systems were used during the research period from October 2004 until May 2007. For ease of reference and simple classification, the ponds were not renamed when research began, and continued to be coded Green and Blue ponds (the names allocated by Abis, 2002). The experimental ponds used are shown in Figure 4.1.

Esholt WWTW is a very large facility (ten hectares) serving a population equivalent of 320,000 people; however, with additional industrial wastes received, this figure increases to 720,000 population equivalents which corresponds respectively to a wastewater comprised of 44.4% of domestic wastewater and 55.6% of industrial wastewater by volume.

Abis (2002) determined the mean BOD, SS and ammonia concentrations entering the three PFP's using data which Yorkshire Water had provided for the two-year period January 1998 and December 1999, and also incorporating data gained from the initial two year operating period of the ponds. The mean BOD value entering the ponds was found to be 485 mg/l and the mean SS value measured 1057 mg/l, and the ammonia concentrations varied seasonally within the range of 18–40 mg N/l (Abis, 2002).

During the experimental period, research was conducted in five main stages; this was determined partially at the outset of the experimental work, but also came about as a direct result of having to replan experimental scheduling when difficulties arose on site.

4.2.1 Modifications to the Esholt WSP systems

A few problems concerning effective operability and sound pond performance became apparent in the initial period after the ponds were inherited, and a number of modifications were undertaken in order to try and remediate the difficulties. Table 4.1 provides the dimensions of each pond.

Table 4.1: Dimensions for the Green and Blue PFP's.

Parameter	Dimensions Green pond	Dimensions Blue pond
Length	9.9 m	9.8 m
Width	3.4 m	4.14 m
Depth	1.5 m	1.5 m
Surface area	33.6 m^2	40.6 m^2
Base area	15.6 m^2	15.4 m^2
Volume	51.3 m^3	58.8 m^3

Figure 4.1: The two primary facultative WSP at Esholt WWTW used over the research period. The pond in the left of the picture is the Blue pond, and the pond in the right, the Green pond.

Firstly, the fresh water used to supply the ponds and control the hydraulic retention time (θ) was supplied by a rudimentary method detailed in Abis and Mara (2005). The freshwater flow was regulated by a crude tap valve and split three ways from a central standpipe, where flows were further controlled by individual diaphragm valves at each pond inlet. Daily losses of flow to the system occurred not only through changes in pressure from the pipe distribution network supplying the standpipe, but also from friction losses and leakage within the feed pipes. The loss in flow to the ponds was further increased because the water had to be pushed uphill over the earthen embankments forming the sides of the PFP's.

To overcome this, a 68-litre Titan cold water storage tank was modified to incorporate the additions of a ballcock and three tap valves, and was placed on breeze blocks on the paved walkway dividing the Red and Green PFP's. Water was then fed to the cold water storage tank from the stand pipe, and the water level within it controlled by the ballcock valve. The water tank system can be seen in Figure 4.1 and is shown in more detail in Figure 4.2. The elevation of the tank provided adequate head pressure, ensuring fresh water flow to the Blue and Green ponds, at a constant rate. The tap valves in the cold water storage tank were simply adjusted to obtain the correct flow. Although somewhat crude, this method proved highly reliable and flows tended to vary by only 100 ml/min between weekly site visits. However, supplying a fresh water flow to the Blue pond, located furthest away from the tank, by the same method was more problematical; a correct flow rate could not be sustained because of the undulating ground on which the pipe was laid, combined with the distance over which the water had to flow. This resulted in large frictional losses within the pipe thus inhibiting flow. When work on the Green PFP terminated, the peristaltic pump which provided the influent wastewater flow was used to supply a fresh water flow to the Blue pond, pumping water directly from the tank to the pond. This worked excellently as both the sewage and fresh water flows could be controlled very accurately, which was crucial for the $^{15}NH_4Cl$ and Rhodamine WT spikes. In order to mitigate the freezing of pipes in the winter, the cold water storage tank was housed in a 182-litre Titan tank, the internal space between the two tanks filled with thick loft insulation, and all external sewage and water pipes lagged with 25-mm thick pipe lagging. After the insulation of all the pipes, the flow of water and wastewater was never compromised by freezing in winter periods, so ensuring that the system ran continually throughout the year.

Figure 4.2: The cold water storage tank used to control the flow of fresh water into the PFP's.

Secondly, the ponds at the time of take over were initially fed according to a BOD of 500 mg/l. This figure was obtained by Abis (2002 − Appendix A) during the two-year start-up period of the ponds, and this value was kept as a constant when determining the loading, and thus the flow, of sewage to the ponds. Subsequent analysis of the weekly grab sewage samples, revealed that often the concentration of BOD delivered to each pond varied between the ponds, as Abis (2002) also found. Not only did this revelation feature significantly in terms of general pond loading, but for long periods of time the BOD in the influent was often well below 500 mg/l. Therefore, in order to make weekly pond loading even more accurate, loading tables were prepared and kept in site books, and the weekly BOD_5 used to determine the loading regime for subsequent weeks, the two methods being used in conjunction gradually to optimise the system. The wastewater and fresh water flows were adjusted according to the BOD_5 value of the previous weeks samples; thus the hydraulics were always kept constant and could be tightly controlled by adjusting the peristaltic pumps and the flow from the water tank. Loading tables and further details of pond loading can be found in Appendix A.

Thirdly, in order to accurately measure the influent flows, V-notches were cut into the side of the 110-mm PVC 'plastidrain' pipe which comprised the inlet structure, into which the reinforced PVC screened sewage and freshwater influent feed pipes were channelled. This enabled the influent feed pipes to be pulled out through the V-notches and the flows measured in this location, rather than taking

the whole pipe out to measure the flow (as was required with the previous system). This was particularly important for the fresh water flows, as taking the feed pipe out of the plastidrain PVC inlet structure caused elevation, and changing the level of the feed pipe hugely affected the flow of water measured by increasing friction within the pipe. The volume of flow measured in this way was wholly inaccurate, and when the pipes were put back into the inlet structure in their normal position, the flow would change dramatically. The modified inlet structure is depicted in Figure 4.3.

4.3 Pond operation and maintenance requirements

Operating and maintaining the PFP's to a high standard was of crucial importance to the efficient and effective smooth running and stability of the systems. This necessitated the minimum requirement of a site visit each week for maintenance work to be attended to.

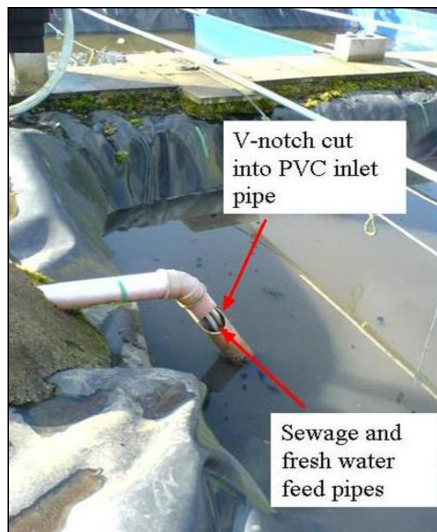

Figure 4.3: An example of the modified inlet structures for the PFP's.

Pond operation and maintenance requirements ensured the following site duties were routinely and systematically followed:

- The wastewater flow was checked in triplicate by a volumetric method (i.e., measuring the flow in a one-litre measuring cylinder for exactly one minute), and each value logged in a site log book and an average taken. The freshwater flow was checked in triplicate by the same procedure, and logged in the same manner; both flows were recorded in ml/min.

- The sewage flow was turned off and the filters scrubbed. If the filters were blocked, they were removed from the pipes and flushed thoroughly to remove debris.
- The sewage flow was turned on again, and allowed to stabilize for at least ten minutes to clear any air locks that may have accumulated in the pipe network from the cleaning process.
- Surface debris such as dead animals, frogs, leaf litter and other floating biomass, like duckweed and various aquatic flora, were removed from the pond.
- The sewage flow was then checked after cleaning the filters and, if necessary, was adjusted to the appropriate flow, or adjusted to a new flow if the BOD_5 had changed. Again, flows were measured in triplicate and logged.
- If the wastewater flow was altered, the freshwater flow was readjusted appropriately, so that the correct hydraulic regime, BOD loading, and hydraulic retention time were maintained. A chart providing sewage and fresh water flows for a specific hydraulic retention time and BOD loading according to the strength of the influent BOD_5 was prepared and kept in the site log book. This was consulted in conjunction with the preceding few weeks BOD_5 measurements, so that on each site visit, the sewage and freshwater flows could be adjusted, if the strength of the sewage measured in the lab by a BOD_5 test proved to have differed significantly between one week and the next. The flows were logged in triplicate and a value take. The changes were seldom, and the flows only ever varied by up to ± 50 ml/min.

4.4 Weekly routine monitoring and sample collection strategy

Weekly or fortnightly grab samples were collected on site typically at around 11.00 am, stored in a cool box, and transported back to the Public Health Laboratories within the School of Civil Engineering, University of Leeds, for analysis. Initially six samples were taken: influent, column and effluent samples for both the Green and Blue ponds. The column sample was taken with a plexiglass 100 mm diameter, 120 cm long column, from the mid-section of the

length of each pond, using the method developed by Pearson *et al.* (1987a). Analysis of all samples began during the afternoon of the day on which they were collected (see section 4.4.1) and, where analysis was still pending, samples were kept overnight in a refrigerator at 4°C for safe storage, and analysed the following day.

From August 2006, when the summer spike was conducted on the Blue pond, influent, column and effluent samples were collected, and seven additional samples also obtained. A T-shaped aluminium rig was fabricated from ‘speed rail’ with a plate running vertically down the central section. The arms of the rig spanned the width of the pond, and the central section extended to the base of the pond. Lengths of 5 mm internal diameter PVC pipe were fixed at set 20 cm intervals from the pond surface to an incremental depth of 20, 40, 60, 80, 100 and 120 cm, down the central section of the rig, to obtain samples from these depths. A SweetWater Microfilter hand pump (MSR, Seattle, USA) with the filtration media completely removed, was attached to each tube in turn at a specific depth, and water pumped into 500 ml HDPE Nalgene sample bottles. A seventh sample was collected form the surface water of the pond by skimming the upper 2 cm of the surface with a sample bottle.

4.4.1 Analytical laboratory methods

This section refers to the analytical laboratory methods conducted on the weekly grab samples collected from Esholt. All samples were analysed according to methods and protocols outlined in *Standard Methods for the Examination of Water and Wastewater*, 20th edition (APHA, 1998), unless otherwise stated.

All samples were analysed for total suspended solids (SS) (method 2540 D, APHA, 1998) using 90 mm diameter Whatman GF/C glass fibre filter papers. Some samples later on in the experimental period were analysed for volatile suspended solids (2540 E). Influent wastewater samples were analysed for settleable solids (2540 F) prior to the suspended solids analysis.

BOD_5 tests were carried out on both raw and unfiltered influent and effluent samples according to method 5210 B (APHA, 1998). Similarly COD (5220 C) was measured on influent and effluent samples and their filtrates. Both BOD_5 and COD tests for each sample analysed were carried out in duplicate. Influent and effluent samples were also analysed in triplicate for their faecal coliform content (9222 D).

Ammonium analyses, conducted on all samples collected within the first year of study, were measured by the ammonia-selective electrode method (4500-NH_3 D). This method often proved problematic and inaccurate for a number of reasons, and the method was therefore discontinued for weekly measurements, and the preferred distillation method substituted (4500-NH_3 B). This method was not without problems, however, and to reduce the accumulation of error throughout the process, all samples were filtered through Whatman GF/C fibre glass filter papers before they were distilled.

Organic nitrogen was measured by the semi-micro Kjeldahl Nitrogen procedure (referred to hereafter as TKN – Total Kjeldahl Nitrogen; method 4500-Norg C) for both filtered and unfiltered samples. The suspended organic nitrogen fraction of a sample was obtained from subtracting the filtered TKN value from the unfiltered TKN value, and the soluble organic nitrogen fraction was obtained from subtracting the ammonium value of that sample from its filtered TKN value.

Sulphide was measured fortnightly in influent, column and effluent samples in order to act as an indicator of sulphide toxicity in the pond. A Hach portable field test kit was used to measure sulphide in situ by the photometric colour measurement (4500-S^2 D), but often this was difficult in practice, as particulates and microbiological constituents within the samples interfered with the readings. Small sample bottles were subsequently fully filled and brought back to the lab, where the sample was immediately centrifuged, and the supernatant taken for analysis by the same field test kit method.

Chlorophyll-*a* and bacteriochlorophyll concentrations were measured by the methanol extraction method according to Pearson *et al*. (1987c) on column, depth,

and effluent samples. Where it was not possible to analyse samples on the same day of collection, samples were filtered and the filtered mass of chlorophyll-*a* captured on a 47 mm diameter Whatman GF/C glass-fibre filter paper which was then wrapped in kitchen foil and stored in a refrigerator until it could be analysed.

Total alkalinity was measured in all filtrates of the weekly grab samples (2320 B).

Dionex DX 500 ion chromatography analyzers (Dionex, Sunnyvale, California) in the School of Civil Engineering, University of Leeds, and later in the School of Geography, University of Leeds, were used to determine nitrite and nitrate concentrations (4500-NO_2^- A and 4500-NO_3^- C), as well as other anions. All analyses were conducted on filtered samples, firstly through Whatman GF/C glass-fibre papers, and, additionally for samples analysed on the Geography Dionex, through 0.45 μm membrane filters.

Microscopic analyses were conducted on all unfiltered column, depth, and effluent samples using an Olympus BHX-2 microscope. The algae and some of the bacteria and protozoa present were identified by using both bright-field and phase-contrast lenses of ×100, ×200 and ×400 magnification. References used to identify microscopic organisms included Belcher and Swale (1978), Bellinger (1980), Canter-Lund and Lund (1995), and Patterson (1998).

4.4.2 Sonde data collection

A multiparameter YSI 6820 model sonde probe (YSI Inc., Yellow Springs, Ohio), which interfaced with a 610–DM display and data logger, was used to measure dissolved oxygen (DO; % and mg/l), specific conductivity, temperature (°C), pH, redox potential (ORP), and Rhodamine data, both for the weekly grab samples and for the spike data.

Sonde readings were taken in conjunction with the collection of weekly grab samples. The effluent readings were always taken first, then a pond depth profile starting at the pond surface, and thereafter at 20 cm intervals to a depth of 120 cm. This was achieved by suspending the probe from a pole, with a hook on the end, of around 1.5 m in length from the mid section of the length of the pond. The last

sample to be read was the influent sample. Readings were always taken in this order to prevent fouling of the probe from particles of sludge at the bottom of the pond adhering to its surface, and the higher strength influent wastewater thus distorting values presented by weaker samples. Data were downloaded to a computer using YSI EcoWatch software, version 3.12.10.

4.4.3 Water temperature measurements

Water temperature was also taken at 20 cm depths from the surface of the pond to a depth of 140 cm, with the reading logged on the hour, every hour for the duration of the sampling period. The logging devices used were DS1921G Thermochron iButtons, which were date- and time-stamped, to record temperatures from a pre-determined initial start; they had the capacity to record data over an 83-day collection period at the set time intervals. The iButtons, each of just over 10 mm in size and less than 5 mm thick, were sandwiched by two thick pieces of plastic film cut to size, and heat-sealed to create an airtight waterproof pocket. Eight iButtons were used per pond profile, and the buttons were hung from the surface of the pond, at each 20 cm intervals to a depth of 140 cm. The sealed pockets were tied to a nylon string at these 20 cm intervals, a weight was tied to the lower end of the string 1.5 m, and a float tied to its surface end next to the first iButton to be placed at the pond surface. The float acted as a buoyancy aid, so ensuring that the string would be taut when the buttons were lowered into the water. After the 83-day data collection period, the buttons were collected, cleaned in the laboratory, and the data downloaded to computer by using iButton TMEX software.

4.5 Stable isotope and Rhodamine WT tracer experiments

Isotopic nitrogen (^{15}N) was introduced to the PFP's as an ammonium salt in the form of ammonium chloride ($^{15}NH_4Cl$), obtained from Cambridge Isotope Laboratories, Andover, Massachusetts. In order to calculate the amount of $^{15}NH_4Cl$ needed for each pond, it was assumed that the pond behaved as a completely mixed reactor. ^{15}N has a natural abundance in the environment of 0.3663% (Junc and Svec, 1958; Hoefs, 1987; Faure and Mensing, 2005); in order to increase this isotopic fraction so that it would be detectable in the pond

effluent, the fraction was doubled from its background level of the in-pond concentration of ^{15}N for the first experimental run of winter 2006. It was a concern after the first experimental run that the concentration of $^{15}NH_4Cl$ in the slug was not high enough and might not be detectable; thus for the second and third experimental runs the concentration of $^{15}NH_4Cl$ was increased. For the experimental runs of summer 2006 and winter 2007 the theoretical natural abundance of ^{15}N in the influent wastewater feed was calculated, and this value doubled to calculate the necessary volume of $^{15}NH_4Cl$ needed for the slug. The pond was simultaneously spiked with a 20% Rhodamine WT standard to obtain the hydraulic characteristics of the pond. Rhodamine WT was used as the chosen dye tracer as it was specifically designed for water tracer studies and is stable in sunlight. It has only a slight tendency to be adsorbed onto sediments and other particles contained within a water body (CTG, 2002); this is a very important consideration with WSP systems, which typically contain very high levels of suspended and settleable solids. It was measured fluorimetrically with the sonde fluorometer. The $^{15}NH_4Cl$ and Rhodamine WT (aq) were dissolved in solution in a 100 ml Duran bottle containing distilled water. The quantities of $^{15}NH_4Cl$ and Rhodamine WT used in each experimental run are presented in Table 4.3. The quantity of Rhodamine WT used in each spike was used to produce an in-pond concentration of 110 μg/l, assuming a completely mixed hydraulic model for the PFP's. All the calculations for $^{15}NH_4Cl$ and Rhodamine WT spike preparation are given in Appendix B.

Table 4.3: Quantities of $^{15}NH_4Cl$ and Rhodamine WT used in each spike preparation.

PFP, spike number, and experimental run	$^{15}NH_4Cl$ (g)	Rhodamine WT (g)
Green pond, spike 1, winter 2006	8.903 g	25.659 g
Blue pond, spike 2, summer 2006	31.398 g	32.357 g
Blue pond, spike 3, winter 2007	31.399 g	32.352 g

4.5.1 Site set-up and spike injection

A few days prior to an experimental spike run, a number of things had to be prepared on site. Firstly, the five sludge buckets were lowered into position, and

the iButtons added to the pond. Secondly, the ammonia volatilization chamber was put into place, and the absorption system fitted together and installed in the pumping shed. The autosampler was set up on the paved walkway between the ponds, and programmed accordingly; the hose of the autosampler was tied to a metal frame to ensure that correct position of the hose was maintained, thus ensuring samples would be collected properly by the autosampler. The autosampler was set to run prior to spike injection, to collect background samples to determine their ^{15}N concentrations. The sonde probe was also installed in the pond effluent point (e) shown in Figure 4.4, and set to log hourly real time measurements of DO (% and mg/l), pH, temperature (°C), ORP and Rhodamine WT concentration by a fitted fluorometer probe. Figure 4.5 depicts some of the items involved in the configuration of the PFP site set up.

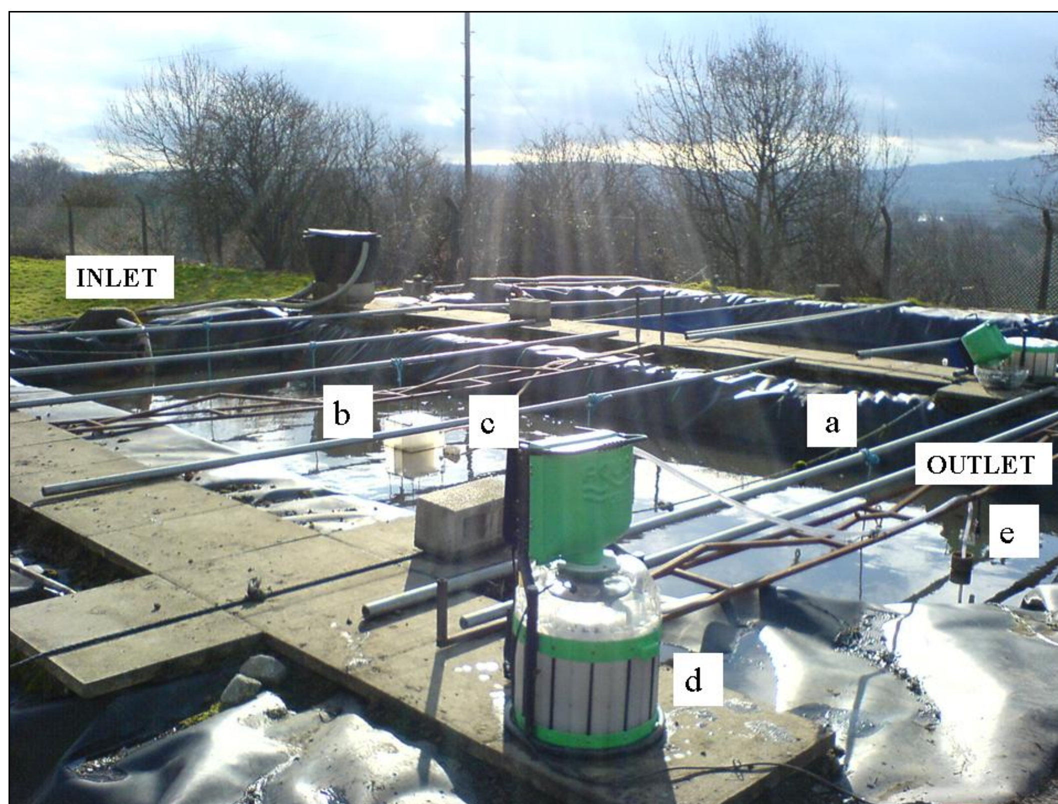

Figure 4.4: A picture showing the experimental set up on site at Esholt, where (a) shows sludge bucket positioning; (b) shows the ammonia volatilization capture chamber; (c) shows the position of the iButton profile; (d) shows the autosampler, and (e) shows the pond effluent point where the autosampler hose and the sonde were placed.

At 11.00 hrs on each designated experimental start date the aqueous spike solution containing $^{15}NH_4Cl$ and rhodamine was introduced into the pond in the influent stream, as an immediate single dose; the single pulse of fluid was poured into a

small funnel connected to influent tubing, and drained into the pond with the influent wastewater and freshwater flows over a 30-second period.

4.5.2 Autosampler collections

An Aquamatic P2-Multiform Aquacell autosampler containing a 24 × 1-litre piece bottler unit, was located on site, adjacent to the effluent point of the pond running the experimental spike. Site visits were made twice a week for spike sample collections, which took place cyclically every three and four days. At the start of the experiment, at the exact time of spike injection into the PFP (i.e., at t_0), the autosampler program was started and pond effluent sampling began immediately. The autosampler had been programmed to obtain a 200 ml sample hourly thereafter. In order to preserve the samples within the autosampler, and prevent ammonia volatilization and any biochemical transformation of nitrogen species, 1 ml of 3M HCl solution containing 1 g of $CuCl_2$/l was added per 100 ml of sample collected within each clean bottler bottle prior to re-starting the autosampler. Samples were transferred on site to specially cleaned 1-litre PET sample bottles for safe storage and conveyance back to the laboratory. The used bottle carrousel was also taken back to the university and soaked in strong disinfectant for 24 hrs, and then hand-scrubbed to remove any biofilm residue and other contaminants. On every site visit a newly cleaned bottler carrousel was fitted to the autosampler and the 3M HCl preservative added to each bottle.

In the laboratory, 1-litre 24-hour composite samples were made from the autosampler collections obtained on site. As a contingency, duplicate composite samples were also made at the same time. The number of samples generated was too prolific to analyse in conjunction with the samples from weekly collections, the ammonia volatilization experiment, additional sample collections and site duties; they were therefore immediately frozen in the laboratory at –20°C until time became available for their analysis.

4.5.3 Laboratory preparation and methodology for ^{15}N extraction

The preparation of samples for mass spectrometry analysis was achieved while simultaneously obtaining the chemical data from each 24-hour composite sample

for suspended organic nitrogen, soluble organic nitrogen, ammonium and nitrate. Samples were partitioned to remove each nitrogen fraction in series; a schematic diagram depicting the stages of sample analysis is shown in Figure 4.6.

Immediately after a sample for total TKN (method 4500 - Norg C, APHA, 1998) had been taken, the suspended organic nitrogen fraction was completely removed by filtration of the sample through 90 mm diameter Whatman GF/C fibre glass filter papers, and the sample analysed for suspended solids (2540 D). Prior to use, all filter papers for ^{15}N analysis were combusted at 550°C for four hours to ensure that any residual contaminants had been removed. The filtrate was collected and a analysed for filtered TKN (4500 - Norg C), ammonium (4500 NH_3 B), and nitrate.

All $^{15}NH_4Cl$ spike samples were analysed for nitrate on a Dionex DX 500 ion chromatography unit (Dionex Corporation, Sunnyvale, California) within the School of Geography, University of Leeds. All samples were prepared and loaded into the Dionex AS50 Autosampler which was coupled to a GP50 gradient pump. A carbonate/bicarbonate mobile phase eluent (strength: 8mM carbonate and 1mM bicarbonate) was prepared daily. Samples were run on an AS14A column with an AG14A guard column to prevent possible contaminants entering the anion exchange resin in the column. Standards of 0.25, 0.5, 0.75, 1, 2, and 4 mg/l NO_2^--N and NO_3^--N were run at the beginning of a sample batch to enable calibration of the data after each run. After the first six standards, the certified reference material, LGC6020 (river water from the River Thames) of 100× dilution and 10× dilution, was included into the sample batch to ensure the validation and accuracy of method. A 1 mg/l standard was run every 10 samples to check for instrument drift.

Samples were run initially using only the ED40 electrochemical detector on a seven minute retention time with a flow rate of 1 ml/min. The results obtained presented difficulties in interpretation, as the high chloride concentrations of the spike samples (see section 4.3.4, where HCl was used for sample preservation within the autosampler on site) considerably interfered with peak separation of nitrate from other peaks; this subsequently affected the integration of the peak area to provide an affirmative result. The action of the chloride produced a highly

fluctuating base line, which also contributed to the difficulty of ascertaining a correct peak area/height from the electrochemical chromatograms produced for each sample run.

To resolve this problem, an AD20 absorbance detector was coupled upstream of the ED40 electrochemical detector, and thus samples were analysed simultaneously by two different methods. As the chloride caused such interference with the nitrate peaks, the method proposed by Raessler and Hilke (2006), for measuring low concentrations of nitrate via UV detection in samples of high salinity, was adopted. Using this method, samples were measured by absorbtion at a wavelength of 210 nm, and an internal nitrate standard was added to each sample to increase the chances of detection and thus measurability. The internal standard prepared was 50 mg/l NO_3^--N/l, and 25 µl added to 2.5 ml of sample, so the ratio of internal standard to sample was 1:100. This ensured a spike of 0.5 mg/l NO_3^--N/l per sample prepared in this way. The eluent rate flow was also reduced to 0.5 ml/min, and the run time per sample increased to twenty minutes, in order to prevent excessive backpressure in the AD20 flow cell.

All anion samples for ion chromatography, collected from the beginning of January 2006, were measured in the School of Geography with the same eluent strength and sample run time, but without the addition of the NO_3^--N spike.

The remaining Whatman GF/C filtered sample (~ 800 ml) was then filtered through C18 Isolute resins containing silica sorbents, which extracted the soluble organic nitrogen fractions by hydrophobic retention mechanisms. A filtration rig, capable of processing four samples simultaneously through the C18 resins, with each unit connected in series, was set up and connected to a diaphragm pump. The C18 Isolute filter apparatus is shown in Figure 4.5.

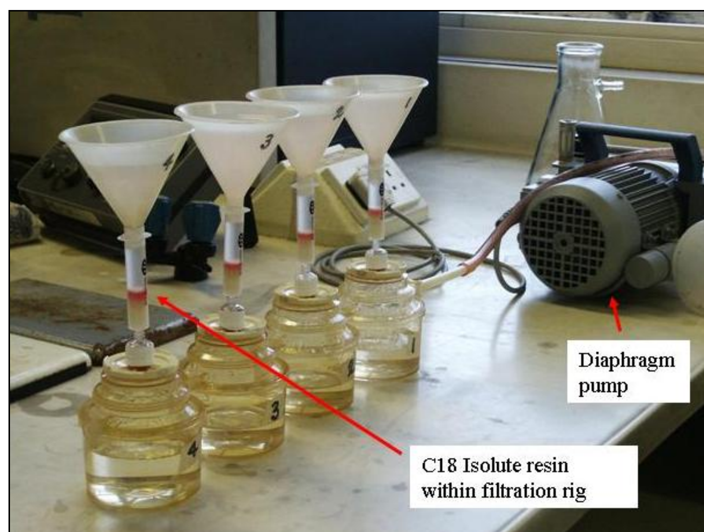

Figure 4.5: The C18 Isolute resin filter rig. N.B.: The pink/brown colouration observable in the middle of the cartridges, are the Rhodamine and soluble organic fractions which have been removed from the sample by the resin.

Upon complete filtration of each sample, the C18 resins were removed, and the filtrate collected for the extraction of ^{15}N from ammonium and nitrate fractions. Two ml of ethanol was pipetted into the C18 resins, and a 60 ml syringe fitted with a rubber bung on the end was connected to the resin cartridge to form an airtight seal. The ethanol eluted the soluble organic nitrogen fraction from the resins. The elution receptacles were 2.0 ml capacity Eppendorf Safe-Lock micro-test tubes, which contained a 2 mm^2 combusted Whatman GF/C glass fibre filter paper acting as an absorption site for the soluble organic nitrogen fraction. After initial elution, C18 resins were wrapped in foil and stored in a container in a fridge. Samples in their respective micro test tubes were put in a sample rack, and left in a fume cupboard for the ethanol to evaporate. Upon complete, or near complete, ethanol evaporation the C18 resin cartridges were again eluted with 2 ml of ethanol, and the ethanol left to evaporate fully this time, so ensuring the Whatman GF/C papers were completely dry. The tube lids were then snapped shut for safe storage of the sample prior to mass spectrometry analysis.

Using a two-stage ammonium diffusion method adapted from Holmes *et al.* (1998), 300 ml of the C18 Isolute filtered sample was transferred into 500 ml Nalgene HDPE bottles, and 2.1 g (a weight derived from the ratio of MgO to sample given in Holmes *et al.*, 1998 − prepared by ashing it at 550 °C for 4 hours) added to the sample to elevate and buffer the sample to a pH of ~9.7. This caused

the ammonium to be converted into ammonia (Holmes *et al.*, 1998), which underwent diffusion and was captured by the ammonia traps.

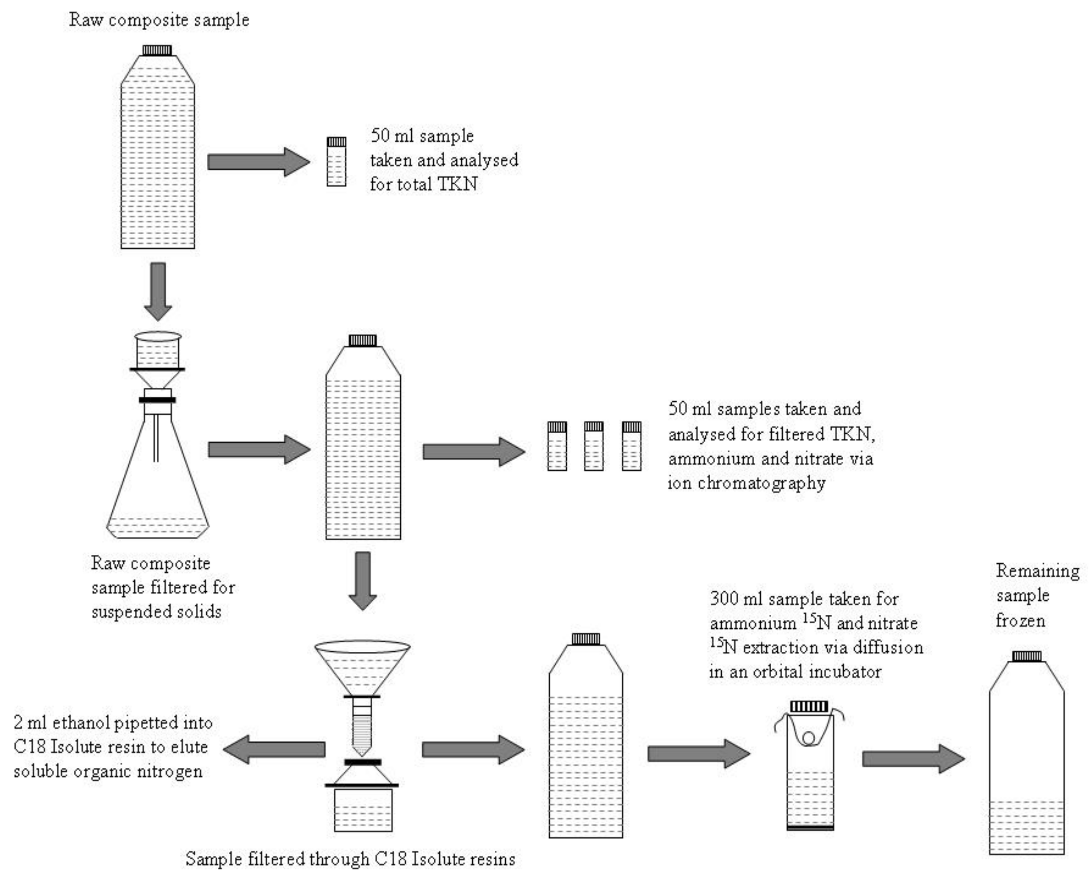

Figure 4.6: Schematic showing ^{15}N sample processing format.

The removal of ammoniacal nitrogen comprised the first stage of the diffusion process. Prior to this stage, two batches of ammonia traps were made using H_2SO_4 to remove the diffused ammonia from the headspace in the bottles via the following reaction:

$$H_2SO_4 + 2NH_3 \rightarrow (NH_4)_2SO_4 \quad (4.1)$$

Tin foil was laid and secured on a lab bench top with paper towels underneath to act as a cushion. The foil was cleaned thoroughly with ethanol and cotton wool; then a series of Millipore Mitex 2.5 cm diameter and 10.0 µm pore size Teflon membrane filters were placed in rows using ethanol-cleaned forceps. A 5 mm diameter bore was used to cut discs of combusted Whatman GF/C papers which

were placed, using forceps, onto the Teflon membranes, and 25 μl of 2M H_2SO_4 were then pipetted onto the discs. A secondary Teflon membrane was laid on top, and the hollow rim of an ethanol-cleaned McCarthy bottle top was used to firmly press the two Teflon membranes together forming a tight seal, thus sandwiching the 2M H_2SO_4 disc in the middle. Two sizes of ammonia trap were fabricated: one held 5 mm diameter GF/C papers for the first diffusion phase which contained higher quantities of ammonia, and the other contained half of a 5 mm diameter GF/C paper with 25 μl of 2M H_2SO_4, to concentrate the reaction area as the concentrations of sample nitrate were much lower than those of ammonia. The traps were stored in air-tight containers until their use. Immediately after the addition of the MgO to the sample, a 15 cm square of mesh fabric gauze was placed over the top of the bottle and pushed down into the bottle to form a cradle, where a larger ammonia trap was placed. The lid was secured immediately, and the sample bottles put in an orbital shaking incubator at a shaking speed of 130 rpm and a temperature of 40 °C. This first diffusion stage took two full weeks, after which time the ammonia traps were removed from the sample bottles. The traps were placed on an ethanol-cleaned metal tray, and the upper Teflon layer carefully pealed from the bottom layer using forceps. In the base of a desiccator below the metal gauze, a 100 ml beaker was filled with 18M H_2SO_4 in order to remove ammonia from the air, creating an ammonia free environment, and the metal tray containing the exposed wet GF/C papers was placed in the desiccator and left to dry for a few days. Following the removal of the ammonia traps for the first stage of the diffusion process, 0.86 g of the reducing agent Devarda's alloy of aluminium, copper and zinc − this weight derived from the ratio of Devarda's alloy to sample given in Brooks *et al.*, 1989) − was added to each sample, which reduced the nitrate to ammonia according to the following reaction:

$$3NO_3^- + 8Al + 5OH^- + 18H_2O \rightarrow 3NH_3 + 8[Al(OH)_4]^- \qquad (4.2)$$

Again, the fabric gauze was placed over the neck of the bottle and a smaller trap placed in the gauze cradle; the lids replaced, and the samples returned to the shaking incubator for another full two weeks. Here nitrate underwent conversion into ammonia, and the ammonia diffused from the liquid to gas phase, where it combined with the sulphuric acid in the ammonia traps, in the same reaction

presented in equation 4.2. Samples from the secondary diffusion stage were dried in the same way as the samples from the first stage; when all samples had dried completely, the GF/C discs were transferred to thoroughly cleaned Bijou bottles, and the lids tightly screwed on.

4.5.4 Nitrogen Stable Isotope analysis

All mass spectrometry analyses were carried out in the School of Earth and Environment within the University of Leeds, in an elemental analyzer (EA) coupled to a continuous flow isotope ratio mass spectrometer (EA-IRMS; Eurovector EuroEA3028-HT elemental analyser and GV Isoprime mass spectromter) to determine the ^{15}N:^{14}N ratio. The mass spectrometer was continually calibrated using internationally certified standards from the United States Geological Survey (USGS), certified by the International Atomic Energy Agency, Vienna. Three standards were used for the calibration: USGS 25 ($(NH_4)_2SO_4$, with a δ ^{15}N value of −30.4‰), USGS 26 (also $(NH_4)_2SO_4$, with a δ ^{15}N value of +53.7‰), and USGS 32 (KNO_3, with a δ ^{15}N value of +180.0‰). Samples and were weighed into small Sn capsules on a micro-balance to three decimal places, according to their estimated nitrogen composition, and their weights recorded. Blanks, standards and samples were loaded in a carefully considered order into the autosampler carousel prior to each batch run; they were sequentially combusted with a pulse of O_2 at the top of the first reagent column, within the first furnace at a temperature of 1020°C. The gas then passed through chromium oxide to complete combustion (Sephton *et al.*, 2002). (Sulphur and halides are scrubbed from the carrier flow by silvered cobaltous oxide within the reagent column.) The flow was passed into a secondary packed reagent tube in a second furnace at 600°C, containing Cu granules, which removed the excess O_2 and nitrogen oxides from the carrier flow (Sephton *et al.*, 2002). Before passage of the flow into the GC within the EA, a CO_2 trap and a H_2O trap were fitted and filled respectively with carbosorb granular and anhydrous magnesium perchlorate.

The abundance of ^{15}N with respect to ^{14}N is expressed in delta (δ) notation; this signifies the difference in ^{15}N:^{14}N ratio between the sample and the international standard for nitrogen (air) in parts per thousand (per mille, ‰), as follows:

$$\delta^{15}N_{Air} = \left[\frac{(^{15}N/^{14}N)_{Sample} - (^{15}N/^{14}N)_{Air}}{(^{15}N/^{14}N)_{Air}} \right] \times 1000 \qquad (4.3)$$

Camargo Valero (2008) formulated an equation which enabled the actual concentration of ^{15}N to be calculated using the $\delta^{15}N$ values for each sample, and the average value of each nitrogen fraction from within an experimental run. This method and the corresponding calculations are presented in Appendix D.

4.6 Ammonia volatilization

The assessment of ammonia volatilization from the PFP's was monitored in conjunction with the Rhodamine WT and $^{15}NH_4Cl$ spikes. An ammonia trap in the form of a capture chamber was partially submerged in the pond, to trap and measure the flux of ammonia-nitrogen being released from the WSP system as gas. The trap used was a transparent five-sided Perspex box (dimensions were 0.34 × 0.33 × 0.48 m) which was fabricated specially for the ponds, and is detailed in the method presented in Epworth (2004).

Initially, the ammonia was scrubbed from the volatilized gas by pumping it through three 250 ml conical flasks in series. The Perspex chamber and boric acid absorbtion system were rigorously tested in the laboratory to ascertain its effectiveness in capturing volatilized ammonia. The system fabricated by Epworth (2004) was only partially effective, however, because not all of the ammonia volatilized from the surface area of water tested could be captured by this method. The whole system, incorporating both capture chamber and ammonia absorbance system, underwent vigorous optimization in the laboratory by Camargo Valero (2007) to produce a sound extraction and measurement system. Baffles were added to the basic box design, and a range of absorbance systems tested over a period of time (Camargo Valero and Mara, 2007). The preferred system incorporated elements of design from other systems used in works such as Zimmo *et al.*, (2003) and Caicedo Bejarano (2005). The final design incorporated the baffled box as the capture system, and a column packed with different graded media, followed by three 250 ml capacity conical flasks run

in series. The full design and operational intricacies of the system are given in Camargo Valero and Mara (2007).

The capture chamber was attached to a pre-fabricated steel ladder-like structure and suspended over the pond, and partly immersed into the water. The absorbance system was constructed in the pump house where the WSP peristaltic pumps were kept. The chosen pump to draw off the ammonia gas from the head space of the capture system was a Charles Austin D Max 200 diaphragm pump. All system components were connected using reinforced PVC tubing. A flow meter was inserted into a section of the tubing after the pump, enabling the flow of air through the pump to be controlled and adjusted. The air flow was kept at 3 l/min for the duration of each experiment. Air stripped from the pond surface, containing ammonia released as gas, was bubbled through a total of 2 l of 2% boric acid which scrubbed the ammonia from the air flow. The packed column contained 1,400 ml of boric acid, and each of the three conical flasks 200 ml. The boric acid in the column and flasks was changed weekly and collected in separate containers, and analysed in the laboratory for ammonia (4500-NH_3 B) against a blank control. A 300 ml weekly composite sample was then prepared and put in the shaking incubator for two weeks, to extract any available $NH_3-^{15}N$, in the same way that spike samples were prepared for $NH_3 - {}^{15}N$ extraction as detailed in section 4.5.3. Results for $NH_3-^{15}N$ were obtained using the mass spectrometer in the School of Earth and Environment (detailed in section 4.5.4).

4.7 Sampling for molecular microbiological analyses

Molecular microbiological analyses were carried out in collaboration with the University of Newcastle. Samples were taken monthly from the influent, column, effluent, and at consecutive 20 cm intervals from the pond surface to a depth of 120 cm. The sludge was also sampled from just below the influent point, from the middle of the pond, and from below the effluent point. During the $^{15}NH_4Cl$ and Rhodamine WT spikes, samples were collected for time sequence analysis a day before spike injection, the day of the spike, one day after, one week after, and then at the end of each of the three hydraulic retention times. Samples were preserved in 50 ml sterilised polypropylene Corning centrifuge tubes on site: 25 ml of the volume was sample, preserved in 25 ml absolute ethanol (1:1 v/v). All of the

molecular microbiological analysis was undertaken within the School of Civil Engineering and Geosciences, University of Newcastle.

Firstly DNA was extracted from selected experimental samples, followed by PCR runs using a variety of primers to target different groups. Following PCR runs, denaturing gradient gel electrophoresis (DGGE) was carried out to establish the number of predominant taxa present within each individual target group. After DGGE analysis, the sequencing of excised bands was conducted and comparisons made between the sequences obtained, and databases in the public domain.

4.8 Sludge sampling and analysis

Primary facultative pond sludge from the Green and Blue pond was sampled every three to six months. Sludge collection devices, in the form of five 10-l metal buckets and five plastic containers, were suspended within the pond from 12 mm polypropylene rope, which was tied to 7 m steel scaffold poles which spanned the width of the pond. Buckets were strategically placed down the centre of the pond between the influent and effluent points. Buckets were placed 1.5 m from the pond influent, then at 3 m, 5, m, 7 m and 9 m along the central line of the pond. After the required time of submergence, the buckets were lifted from the PFP's and the contents allowed to settle for over an hour on the bank of the pond. Then the supernatant was poured off until the sludge started to waste from the bucket; the sludge was then poured into a suitably sized container and brought back to the laboratory for analysis. Buckets were thoroughly rinsed, and re-tied to the scaffold poles and lowered into position within the pond once again.

4.8.1 Sludge analysis

Primary facultative pond sludges were firstly settled in the lab in 1-litre Imhoff cones and measuring cylinders. In these, following complete settlement, the volume of sludge was recorded, the supernatant disregarded, and the sludge stored in a separate container, until the total volume collected from site had been treated in this way. Sludge from each separate batch was analysed by the selective ion electrode method for ammonia determination (method 4500-NH_3 D), and in duplicate or triplicate for TKN (4500-Norg C). Total solids dried at 103−105°C

(2540 B), and fixed and volatile solids (2540 E) were measured in duplicate. A portion of sludge from each batch was dried at room temperature and also analysed for ^{15}N content by the mass spectrometer.

4.8.2 Sludge depth profiles

The sludge depth within each pond was measured at the start of the whole experimental period, and then every six months thereafter, until a spike experimental run. The depths were again recorded after each experimental run. The method developed by Malan (1964) using a pole with white towel tightly wrapped around it, the so called "white towel test", was used to measure the depth of sludge accumulation at strategically placed points of a grid system, thus allowing the intersections to be used as accurate coordinates for the precise measurement which was recorded. The black crosses in Figure 4.7 show the locations where the sludge depth profiles were taken.

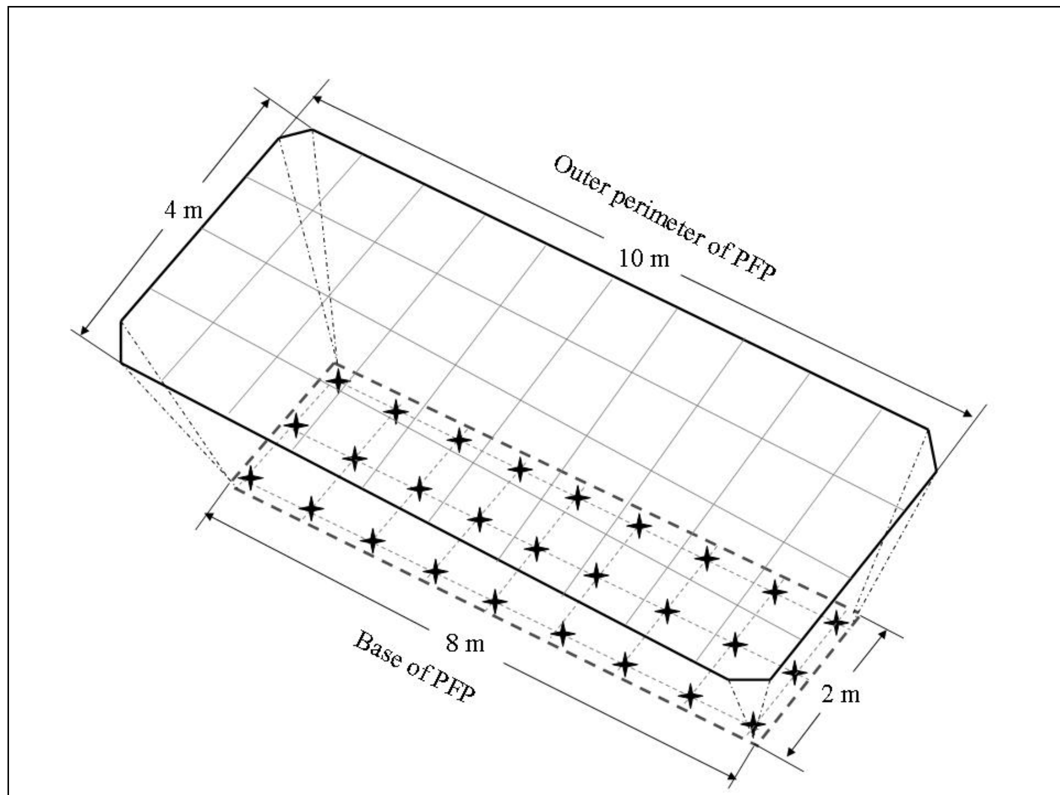

Figure 4.7: A diagram showing how the PFP's were divided into grid at the ponds surface, and the exact location where sludge depth profiles were taken on the base of the pond. NB: drawing not to scale.

A total of 27 readings were recorded for each pond, where each pond was divided into grid comprising 3 × 9 interception points. At each interception point a depth

profile was obtained using a 2.5 m pole and the sludge depth measured using a standard tape measure, as can be seen in Figure 4.8.

Figure 4.8: Measuring the sludge depth profile