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WASTE

The Waste Section of the Environmental RTDI Programme addresses the need for research in Ireland to inform policymakers and other stakeholders on a range of questions in this area. The reports in this series are intended as contributions to the necessary debate on waste and the environment.

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Executive Summary

This project set out to examine the use of biological-based approaches to facilitate the removal of nutrients, particularly phosphates, from food processing waste streams. The work aimed to develop biological management procedures, incorporating molecular-based methodologies to assess the performance of biological nutrient removal (BNR) systems treating these food wastes. To this end, culture-independent molecular-based approaches have been employed to show that in laboratory-scale enhanced biological phosphate removal (EBPR) processes significant changes in the overall microbial community structures can be linked to a failure to efficiently remove phosphate. From this work, it appears that the key factors that influence decreases in phosphate removal efficiency are likely to be changes in the influent composition and, in particular, the quality and quantity of short-chain fatty acids (SCFAs). While further work will need to be undertaken, it appears that the control of influent quality may be a key factor to aid in the successful removal of phosphate by EPBR systems and failure to ensure sufficient levels of SCFAs may be a contributory factor in their malfunctioning.

This project also set out to investigate the use of low pH ‘shock’ as a novel method to remove phosphate from food processing waste streams. Work with synthetic waste streams and laboratory-scale bioreactors operating at a low pH indicated that acidification of the influent to pH 5.2 had the effect of achieving a low reactor pH with a concomitant 50% phosphate removal. Molecular-based approaches identified a number of microbial strains from genera that have recently been reported as having a crucial role in phosphate removal in several activated sludge environments. It is clear, however, that if the low pH ‘shock’ approach is to be useful it will be important to achieve microbial control in feed reservoirs via reduced pH. If influent nutrient concentrations are contaminated particularly with lactose-utilising micro-organisms, then it is likely that insufficient chemical oxygen demand (COD) may be present in the reactor to maintain the sludge sufficiently to promote effective phosphate removal. Future research in this ‘acid shock’ area should investigate the potential to acclimatise sludges with increasing COD concentrations. It is, however, likely that the treatment of industrial waste-water COD will require the incorporation of a pre-EBPR reactor sludge-exposure step to mimic, in a controlled fashion, the effects of reservoir contamination which we have observed in this study.

The project also set out to ascertain, using compost-quality testing standards, whether sludge generated from biological phosphate removal (BPR) could be composted to a satisfactory quality. The project succeeded in establishing and standardising a number of tests and methods to assess compost quality and subsequently evaluated these methods in laboratory-scale test compost units. These methods included inductively coupled plasma–mass spectrometry (ICP-MS), total Kjeldahl nitrogen determination and most probable number (MPN) analysis. Analysis of the laboratory-scale test compost units showed the benefits of monitoring the process using the validated testing methods.

Finally, the project set out to study the performance of a pilot-scale BNR system, operating under a variety of different conditions. This study found the pilot-scale system capable of treating the elevated nitrogenous loading to the plant during the peak season and also capable of performing reliably in the colder off-peak season. Reductions in the size of the anaerobic and anoxic zones, relative to the total size of the mixed reactor zones, together with reductions in the ratio of return activated sludge to influent flow rate, were found to have a beneficial effect on the BNR process, with the activity of the bacteria within the system in terms of COD, NH_4-N and P removal being enhanced by these reductions.

1 Introduction

One of the important factors affecting water quality is the enrichment of nutrients in waterbodies (Romanski *et al.*, 1997). Discharging waste water with high levels of phosphorus (P) and nitrogen (N) can result in eutrophication of receiving waters, particularly lakes and slow-moving rivers (Sundblad *et al.*, 1994; Danalewich *et al.*, 1998). Biological processes are a cost-effective and environmentally sound alternative to the chemical treatment of waste water (Osee Muyima *et al.*, 1997). Currently, the majority of treatment plants incorporating P removal utilise chemical precipitation using alum or lime. Biological removal systems are, however, increasingly being incorporated in sewage treatment works (Stratful *et al.*, 1999). Biological phosphorus removal (BPR) from waste waters is based on the enrichment of activated sludge with phosphate-accumulating organisms (PAOs) (Brdjanovic *et al.*, 1998; Wagner and Loy, 2002). BPR exploits the potential for micro-organisms to accumulate phosphate (PO_4) (as intracellular polyphosphate) in excess of their normal metabolic requirements (Brdjanovic *et al.*, 1998; Mino *et al.*, 1998). The BPR process is primarily characterised by circulation of activated sludge through anaerobic and aerobic phases, coupled with the introduction of influent waste water into the anaerobic phase (Wagner and Loy, 2002). In the anaerobic phase, sufficient readily degradable carbon (C) sources, such as volatile fatty acids (VFAs), must be available, which induce the phosphate-removing bacteria to take up the acids and release PO_4 into solution (Morse *et al.*, 1998). In the aerobic phase, luxury P uptake occurs, which results in overall P removal rates of as much as 80–90% (Morse *et al.*, 1998). A high P removal efficiency can be achieved by wasting excess sludge with high P content (Mino *et al.*, 1998). Incorporation of an anoxic phase permits the combined removal of N and PO_4 from the process waste waters (Metcalf and Eddy, 1991; Crites and Tchobanoglous, 1998). The microbiology and biochemistry of the BPR process have been extensively reviewed by Mino *et al.* (1998). In addition, the discovery of denitrifying PAOs has been extensively reported and discussed (Rensink *et al.*, 1997; Hu *et al.*, 2002). It is believed that, given the appropriate conditions, different species of PAOs, which accomplish anoxic P uptake will find a niche in these systems but will have a significantly low BPR performance and use influent readily biodegradable chemical oxygen demand (COD) less 'efficiently' compared with the aerobic P-uptake PAOs (Ekama and Wentzel, 1999).

Given the fact that enhanced biological phosphate removal (EBPR) or biological phosphate removal (BPR), which exploit the potential for micro-organisms to accumulate PO_4 in excess of their normal metabolic requirements, has successfully been applied in a number of European countries to reduce PO_4 levels in municipal waste-water treatment systems (Mulkerrins *et al.*, 2004), this project set out to examine the feasibility of using this technology with sludges from the dairy industry. Previous work had demonstrated an ability of sludges from dairy waste streams to perform EBPR (Primus, 1998), and other researchers had reported acid-stimulated luxury PO_4 uptake in municipal waste-water treatment systems (McGrath *et al.*, 2001). With this in mind this project set out examine the potential of using both EBPR and acid-stimulated PO_4 uptake as novel methods/technologies for PO_4 removal from food industry waste-water treatment systems.

1.1 Milestones

1. Conduct molecular ecology studies on an EBPR process to study the microbial diversity of the system and to develop biomarkers to monitor the functioning of the system.
2. Investigate acid-stimulated phosphate removal and phosphate accumulation by activated sludges from food industry waste streams.
3. Investigate the feasibility of using sludge generated in a biological phosphate removal plant as a feedstock for compost.
4. Investigate the key operational parameters involved in the biological nutrient removal (EBPR process), while treating a food processing waste-water stream.

1.2 Project Overview

A key environmental problem facing the Irish food sector is the treatment of waste streams generated from the processing of various foods. In particular, the treatment of

P-rich waste streams is very problematic, given the strict maximum levels for waste-water treatment plant effluents set out by the Statutory Instrument No. 258 in 1998 (Local Government (Water Pollution) Act 1997 (Water Quality Standards for Phosphorus) Regulations). These limits were set and are enforced due to the potential problems associated with P release into waste streams, particularly eutrophication. Eutrophication of freshwater environments occurs due to the overgrowth of algae and macrophytes and poses the single biggest threat to water quality in Ireland. Effluents containing elevated P levels from the food industry typically undergo chemical treatment to reduce the discharge P levels. This process, while successful, is expensive and leads to increases in sludge volumes, with accompanying additional disposal problems. A viable alternative to chemical precipitation is that of EBPR which exploits the potential for micro-organisms to accumulate PO_4 to levels that are clearly in excess of their normal metabolic requirements. The EBPR process involves an anaerobic zone and a subsequent aerobic zone which selectively enriches for micro-organisms that are capable of biologically removing PO_4 from waste waters. These EBPR systems have been used at many waste-water treatment plants all over the world for many years (Heinzmann, 2005; Tykesson *et al.*, 2006). In addition, researchers at Queen's University Belfast have reported an acid-stimulated luxury PO_4 uptake by environmental micro-organisms (McGrath *et al.*, 2001). They reported that bacterial cultures displayed an increased ability to take up PO_4 when the pH of the growth medium was reduced from 7.5 to 5.5. Enhanced PO_4 removal by these cultures was accompanied by increases of between 2- and 10.5-fold in their polyphosphate content. Thus, this project set out to establish whether both the EBPR and the acid-stimulated PO_4 removal systems could be used as novel processes to treat high-PO_4 waste streams from the food processing industry.

Specifically, the project set out to develop culture-independent molecular-based methodologies to assess the performance of BPR systems treating waste streams from food processing plants, using both the anaerobic and aerobic schemes typical of the EBPR systems and the 'acid shock' system.

Another goal of the study was to establish and standardise a number of tests and methods to assess compost quality, and subsequently to evaluate these methods in laboratory-scale test compost units. The overall aim was to determine whether sludges generated from EBPR effluent plants could be successfully composted to a satisfactory quality. The final goal of this study was to evaluate the performance of a pilot-scale biological nutrient removal (BNR) system, operating under a variety of different conditions, which had previously been successfully established under laboratory-based conditions in sequencing batch reactors (SBRs). This would establish whether or not the system could successfully function under standard operating conditions in a food industry setting.

2 Project 1: Conduct Molecular Ecology Studies on an EBPR Process to Study the Microbial Diversity of the System and to Develop Biomarkers to Monitor the Functioning of the System

Even though BNR systems offer many advantages over chemical precipitation methods, these systems are not without their own problems. Waste-water treatment plants that run under BNR conditions are vulnerable to system failure. The main reason for this is because little is known about the micro-organisms that carry out the processes or indeed which micro-organisms are responsible for nutrient removal. In the absence of this information, treatment plants are often designed in an empirical manner and operated under conditions that may or may not be suitable for the micro-organisms in question. In these circumstances, it is impossible to predict the effects that changes in reactor operation will have on the micro-organisms in the system and what the resultant reactor performance will be. Thus, it is imperative to be familiar with the micro-organisms that carry out BNR, not only to provide them with optimal conditions for growth, but also to anticipate problems that may be experienced should these micro-organisms be subjected to fluctuations in reactor operation. Monitoring these populations has proven difficult, as many of them cannot be isolated or studied using traditional culturing techniques. However, the past few decades have seen the development of a myriad of molecular techniques that allow the study of complex microbial communities without the need for culturing. These techniques, based on the sequence analysis of the 16S/18S rRNA or functional genes, have frequently been used to examine the microbial diversity of environmental samples or to monitor changes in microbial community structure. The populations involved in both aerobic and anaerobic treatment systems have been widely studied using these methods (Lee *et al*., 2003, 2004). Significant strides are being made into unravelling the microbial communities that are responsible for waste-water treatment. One of the most valuable techniques used in monitoring microbial population changes is denaturing gradient gel electrophoresis (DGGE). This technique not only fingerprints changing microbial communities, but can also provide sequence information on the micro-organisms that are present in any given sample. One of the most important factors for stable waste-water treatment is a stable waste water, as sudden changes or fluctuations can severely compromise process efficiency (Mulkerrins *et al*., 2004). Micro-organisms in laboratory-scale reactors that are receiving synthetic waste waters are cultivated under controlled conditions, where most parameters (pH, temperature, influent composition, etc.) remain relatively stable. Full-scale BNR systems are not stable. As well as being influenced by outside pressures (e.g. temperature, presence of higher organisms, etc.), the micro-organisms in these reactors are subjected to constant fluctuations in reactor influent, the composition and strength of which may change depending on production level. In such a changeable environment, it is easy to see why these reactors are prone to failure. However, if the effects of a reactor disturbance are known, efforts may be made to alleviate similar problems that may occur in the future.

The main objective of this part of the study was to operate a continuous anaerobic/anoxic/oxic (A^2/O) reactor and to monitor shifts in the reactor populations at various stages during the trial. Synthetic waste waters may not reflect either the complexity of 'real' waste waters or the fluctuations to which the micro-organisms in full-scale treatment systems are subjected. The treatment of dairy-processing waste waters is reported to be problematic due to variations in flow and waste-water characteristics (Danalewich *et al*., 1998). Therefore, it was decided to operate the reactor using dairy-processing waste water as influent. During the course of the trial, changes were made to the operation of the reactor and the effects on the reactor populations were analysed using DGGE. A significant change was observed in the DGGE profile of the reactor at two time points and this correlated with the failure in the BNR system to efficiently remove P from the influent. Furthermore, ^{1}H NMR spectra of the BNR system at these two time points revealed a significant difference in VFAs, a factor which is likely to have contributed to the overall failure of the BNR.

2.1 Materials and Methods

2.1.1 Biological nutrient reactor set-up and operation

An A^2/O reactor, with a working volume of 5 l, was seeded with sludge from the pilot-scale A^2/O laboratory-scale bioreactor treating a dairy-processing waste water. The reactor was operated with a 24-h hydraulic retention time (HRT), and an initial sludge retention time (SRT) of 17 days was maintained by wasting an appropriate quantity of sludge each day. The reactor was fed with dairy-processing waste water with average influent COD, PO_4 and NH_4 concentrations of 1,067, 29 and 40 mg/l, respectively. The average influent COD, PO_4 and NH_4 influent concentrations during each SRT period are presented in Table 2.1. After 58 days the SRT was reduced to 12 days, but was increased again to 17 days on Day 85 following a decrease in the P removal efficiency. The reactor was operated for a total of 122 days.

2.1.2 Chemical analysis of biological nutrient reactor

The reactor was sampled three times weekly for COD, PO_4 and NH_4 removal, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), dissolved oxygen (DO) and pH. The COD, PO_4, NH_4 and NO_3 concentrations within the reactor were measured using Hach Methods 8000, 8114, 8038 and 8171, respectively, for water analysis (Hach Company, Loveland, CO, USA). MLSS and MLVSS measurements were determined according to standard methods (APHA, 1992). Dissolved oxygen and pH measurements were determined using DO and pH probes and a WTW meter.

2.1.3 Isolation and PCR amplification of partial 16S rRNA gene products

Biomass samples were taken from the aerobic stage of the reactor following each SRT. A total of eight samples were taken from the reactor, and included in the seed sludge, and biomass from the reactor was sampled on Days 22, 41, 58, 73, 85, 100 and 122 of the trial. DNA from each sample was extracted using a combination of chemical and physical methods. Briefly, 500 mg biomass were subjected to bead-beating for 30 s with 1 g of 0.1-mm sterile zirconium beads, 500 µl PO_4 buffer, 500 µl 10% SDS and phenol/chloroform/isoamylalcohol (25:24:1). Three bead-beating steps were performed, with 5-min incubations on ice in between. Tubes were centrifuged at 16,000 × *g* for 10 min and the supernatant transferred to a fresh tube. Isopropanol (0.7 vol.) was added to each tube and, following a 10-min incubation on ice, tubes were centrifuged at 16,000 × *g* for 10 min. The resulting pellet was washed with 70% ethanol (ice cold), air-dried and resuspended in TE buffer (pH 8.0).

PCR amplification of the V6–8 region of the 16S rDNA gene was achieved using primers 968f (5' AAC GCG AAG AAC CTT AC 3' with a GC clamp (5' CCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCG CCCG 3')) and 1378r (5' CGG TGT GTA CAA GGC CCG GGA ACG 3'), with the extracted DNA serving as a template for the PCR. Each reaction contained 200 ng template DNA, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1 × reaction buffer (20 mM Tris pH 8.4, 50 mM KCl), 10 pmol each primer and 2.5 units of *Taq* polymerase (Bioline), in a final reaction volume of 50 µl. The PCR amplification was carried out in a PTC-100 thermocycler with the following cycles: initial denaturation at 94°C for 2 min, 10 cycles of denaturation (94°C for 1 min), touchdown annealing (1 min starting at 60°C, decreasing 1°C with each cycle) and extension (1 min at 72°C). These cycles

Table 2.1. Average COD, PO_4 and NH_4 concentrations during the trial. Each time period corresponds to one SRT.

Time period (days)	Average influent COD (mg/l)	Average influent PO_4 (mg/l)	Average influent NH_4 (mg/l)
I (0–22)	905	30	43
II (23–41)	912	29	35
III (42–58)	1,340	26	42
IV (59–73)	1,460	32	44
V (74–85)	1,040	31	44
VI (86–100)	920	27	35
VII (101–122)	893	28	39

were followed by 20 cycles of denaturation (94°C for 1 min), annealing (1 min at 50°C) and extension (1 min at 72°C), with a final extension at 72°C for 5 min. Amplification products were checked by electrophoresis of 5 µl of the PCR product on a 1% w/v agarose gel in 1 × TAE buffer and visualised under UV illumination.

2.1.4 DGGE to determine changes in the bacterial community

DGGE was carried out using a DGGE-1001 electrophoresis system (CBS Scientific) at 80 V and 60°C for 18 h. Samples were loaded on a 4% polyacrylamide gel (37.5:1 acrylamide–bisacrylamide) in 1 × TAE buffer. Mixing two stock solutions of polyacrylamide containing 50% and 65% denaturant concentrations formed the denaturing gradient in the gel (denaturing acrylamide of 100% was defined as 7 M urea and 40% v/v formamide). Following electrophoresis, the gels were rinsed briefly in distilled water, stained with SYBR Gold (Invitrogen, Ireland) for 20 min and bands were visualised under UV illumination.

2.1.5 Isolation and sequencing of DGGE bands

Bands in the DGGE gels were excised from the gel using sterile tips and the piece of excised gel was placed in a sterile 1.5-ml microfuge tube. The DNA was eluted from the gel by adding 10 µl of sterile water and placing the tube at 4°C overnight. Bands were re-amplified as described above and run on a second DGGE gel. This procedure was repeated until only a single band was visible on the gel. Bands for sequencing were cleaned using the PCR clean-up kit (Qiagen, UK) and sequenced using the reverse primer 1378r on an automated sequencer (MWG Biotech, Germany).

2.1.6 Cloning library generation and screening

Prior to cloning, the 16S rDNA genes from samples 73 and 85 were amplified by PCR using the forward primer 27f (5' AGA GTT TGA TCM TGG CTC AG 3') and the reverse primer 1492r (5' GGT TAC CTT GTT ACG ACT T 3'). Each reaction mixture contained 200 ng DNA, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1 × reaction buffer (20 mM Tris pH 8.4, 50 mM KCl), 10 pmol each primer and 2.5 units *Taq* polymerase (Bioline, UK) in a final reaction volume of 50 µl. Clone libraries were generated for these samples by ligating the 16S rRNA partial gene products from this PCR amplification into the TOPO-TA cloning vector and transforming chemically competent *Escherichia coli* One Shot TOP10 cells as per the manufacturer's instructions. Clones were screened for the correct sized insert by amplifying the insert using M13 primers and running the products on a 1% agarose gel, followed by UV illumination. The correct sized inserts were restricted using the tetrameric restriction endonuclease *Hind* III incubated at 37°C for 2 h. These restriction reactions were electrophoresed on 3% w/v agarose gels producing restriction fragment patterns. These patterns were analysed by eye and grouped into operational taxonomic units (OTUs). A representative clone of each OTU was sequenced using the reverse primer 1492r on an automated sequencer (MWG Biotech, Germany).

2.1.7 Phylogenetic analysis of retrieved sequences

Alignments of the 16S rRNA gene were made using sequences downloaded from the Ribosomal Database Project II (RDP II) (Cole *et al*., 2005) after searching for its nearest neighbours using the sequence match tool. Further searches were conducted using BLAST (Altschul *et al*., 1997) and FASTA (Pearson *et al*., 1988) of the EMBL (Cochrane *et al*., 2006) database for further 16S rRNA sequences which are closely related to the isolates' 16S rRNA, but are not available from the RDP II. The RDP sequences were used as the reference set and the 16S rRNA sequence of the OTUs and any other closely related 16S rRNA sequences obtained from EMBL were aligned to the references data set using the profile alignment option in CLUSTAL_X (Thompson *et al*., 1997). The resulting alignment was further refined using BioEdit ver. 7.0.5.2 (Hall, 1999), and subsequently parsed through Gblock (Castresana, 2000) in order to remove ambiguously aligned sections and increase the robustness of the data. Using MEGA version 3.1 (Kumar *et al*., 2004), a global phylogenetic tree was determined using the neighbour-joining (NJ) method. Evolutionary distances for the global tree were calculated using the Kimura-2-parameter (K2P) model with a transition/transversion ratio of 2. Further trees were constructed, with the PHYLIP package (Felsenstein, 1993) in BioEdit, using maximum parsimony and maximum likelihood methods to determine the position of the various OTUs' 16S rRNA sequence. All NJ trees were tested statistically by means of bootstrap analysis using MEGA version 3.1.

2.1.8 ^{1}H NMR spectroscopy of waste water

Samples for NMR analysis (for Days 73 and 85) were prepared by mixing 400 ml of waste water with 200 ml of

PO_4 buffer containing 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4, 10% D_2O as a field lock and 0.05% sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 as a chemical shift reference (pH 7.4). ^1H NMR spectra were recorded on a Bruker DRX 600 NMR spectrometer equipped with a Bruker 5-mm triple resonance with inverse detection probe, operating at 600.13 MHz for ^1H. A standard one-dimensional pulse sequence (RD-90°-t_1-90°-t_m-90°-ACQ) was employed. Water suppression was achieved with weak irradiation on the water peak during the recycle delay (RD = 2 s), and mixing time, t_m, of 100 ms and t_1 was set to 3 ms. The 90° pulse length was adjusted to approximately 10 ms. Sixty-four transients were collected into 32 k data points for each spectrum with a spectral width of 20 ppm and a total repetition time of 3.8 s. Spectra were corrected for phase and baseline distortions and referenced to sodium 3-(trimethylsilyl)-propionate-2,2,3,3-d4. A line-broadening factor of 0.3 Hz was applied to the spectra prior to Fourier transformation. For assignment purposes, two-dimensional (2D) correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) NMR spectra were also acquired for the waste water; details have been reported previously (Wang *et al*., 2006).

2.1.9 Profile analysis of DGGE patterns

DGGE profiles were analysed using the Gel Compar II (Applied Maths, St-Martens-Latem, Belgium). Similarities between samples were determined by calculating similarity indices based on the DICE similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA). The DICE coefficient is also referred to as Sorenson's pairwise similarity coefficient (C_s) and is commonly used to compare species composition of different ecosystems. Two identical profiles create a value of 100%, whereas two completely different profiles result in a value of 0%. Dendrograms of DGGE banding profiles were constructed to visualise any clustering patterns evident and to generate similarity matrixes for numerical and subsequent statistical analysis. All similarity results given are the DICE and UPGMA percentage similarities as this method (band based) and Pearson correlation coefficient (curve based) were in agreement (data not shown). Composite data sets for DGGE profiles were generated in Gel Compar II and numerical band matching character tables produced for export and analysis by BiodiversityPro (version 2, Scottish Association for Marine Science, http://www.sams.ac.uk. Using the BiodiversityPro software Simpson's, Shannon–Weaver and Fisher's alpha ecological indices of diversity were generated. These were calculated using the following equations:

$$\text{Simpson's index (D)} = \sum_{i=1}^{s} \frac{n_i(n_i-1)}{N(N-1)}$$

where n_i is the the total number of organisms of the *i*th species or band intensity and N is the total number of organisms of all species or total band intensities (Simpson, 1949).

$$\text{Shannon–Weaver index (H')} = -\sum_{i=1}^{s} (P_i)(\ln P_i)$$

where s is the number of species/bands in the sample and P_i is the proportion of species/bands for the *i*th species/band in the sample (Shannon and Weaver, 1949; Boon *et al*., 2002).

$$\text{Fisher's alpha index } (\alpha) = \frac{N(1-x)}{x}$$

where x is calculated from

$$\frac{S}{N} = \frac{(1-x)}{x(-\ln(1-x))}$$

and S is the number of species/bands and N is the total number of individuals/bands (Fisher *et al*., 1943; Kempton and Taylor, 1974).

2.1.10 Statistical analysis

All percentage similarities and diversity indices were analysed to determine whether it was normally distributed (using SPSS probability plot (P-P) function). For normal data unpaired Student's *t*-tests were performed while for non-normally distributed data Wilcoxon's test was applied.

2.1.11 Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study have been deposited in the EMBL database under accession numbers AM268117 to AM268137 for the 16S rRNA clones OTU 1–21 and AM268138 to AM268149 for the 16S rRNA, bands 1–12, sequences derived from the DGGE profiles.

2.2 Results

2.2.1 Reactor performance

The average influent COD, PO_4 and NH_4 concentrations during each SRT period are presented in Table 2.1. It is evident that the composition of the waste water fluctuated during the trial period. Both influent PO_4 and NH_4 concentrations remained relatively stable throughout the trial, but COD concentrations varied considerably. The reactor quickly achieved stable operational performance with respect to NH_4 and COD removal, with average NH_4 and COD removal efficiencies of 99% and 91%, respectively, being achieved during the start-up period (Period I; Table 2.2). The process of PO_4 removal took longer to become established in the reactor, and an average removal efficiency of just 61% was achieved during the start-up period (Table 2.2 and Fig. 2.1). Phosphate removal gradually improved from Day 33 until Day 59, when a maximum removal efficiency of 89% was achieved.

From Day 22 to Day 59, the SRT was maintained at 15–17 days and the reactor performance continued to improve during this time. While NH_4 removal remained constant, the COD removal efficiency increased from an average of 94% during Period II to 97% during Period III. Removal of PO_4 decreased to 60% during Period II, but showed a marked increase to 74% during Period III (Table 2.2). The improvement in PO_4 removal may be due to higher COD concentrations that were present in the influent during this time (Table 2.1), which is known to be an important factor in PO_4 removal (Mulkerrins *et al.*, 2004).

It has been reported that an SRT of 10–12 days is optimal for both N and P removal (Chuang *et al.*, 1997; Kargi and Uygur, 2002); thus the SRT was reduced on Day 59 from

Table 2.2. Average COD, PO_4 and NH_4 removal efficiencies during each time (SRT) period.

Time period (days)	Average % COD removal efficiency	Average % PO_4 removal efficiency	Average % NH_4 removal efficiency
I (0–22)	91	63	99
II (23–41)	94	60	99
III (42–58)	97	74	99
IV (59–73)	97	77	99
V (74–85)	95	59	99
VI (86–100)	96	47	99
VII (101–122)	95	46	99

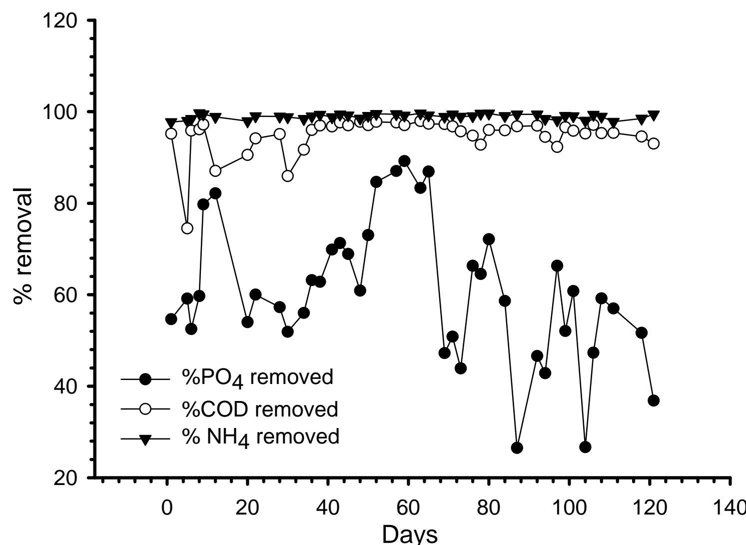


Figure 2.1. Performance of the A^2/O reactor with respect to PO_4, COD and NH_4 removal.

17 to 12 days. Following this change, the PO_4 removal efficiency continued to improve, averaging 77% during Period IV (Table 2.2) and a maximum PO_4 removal efficiency of 89% was recorded on Day 59 (Fig. 2.1). The reactor maintained its stable performance with respect to COD and NH_4 removal.

A marked decline in reactor performance was noted during the subsequent periods of operation. While COD and NH_4 removal remained largely unaffected, the average PO_4 removal efficiency decreased to 59% during Period V and further to 47% and 46% during Periods VI and VII, respectively. It was believed that the change in SRT may have effected this change in reactor performance and so the SRT was increased to 15 days on Day 85. However, the reactor performance continued to decline and operation was ceased on Day 122.

2.2.2 *Microbial community changes as determined by analysis of the DGGE profiles*

PCR-DGGE was used to compare the bacterial community structure in the reactor throughout the trial. The DGGE profiles of the seed sludge and the biomass samples taken from the reactor following each SRT are shown in Fig. 2.2. Each of the samples showed considerable diversity, with many bands present in each profile, even though some were quite weak. It can be seen that, although slight differences are evident between the seed sludge and the biomass sampled following Periods I, II, III and IV (Fig. 2.2, Lanes 1–5), the changes are minimal and numerous bands are common to all five samples. This observation implies that the waste-water constituents and operational conditions were relatively stable during this period. However, the differences between these samples and the profiles of samples taken

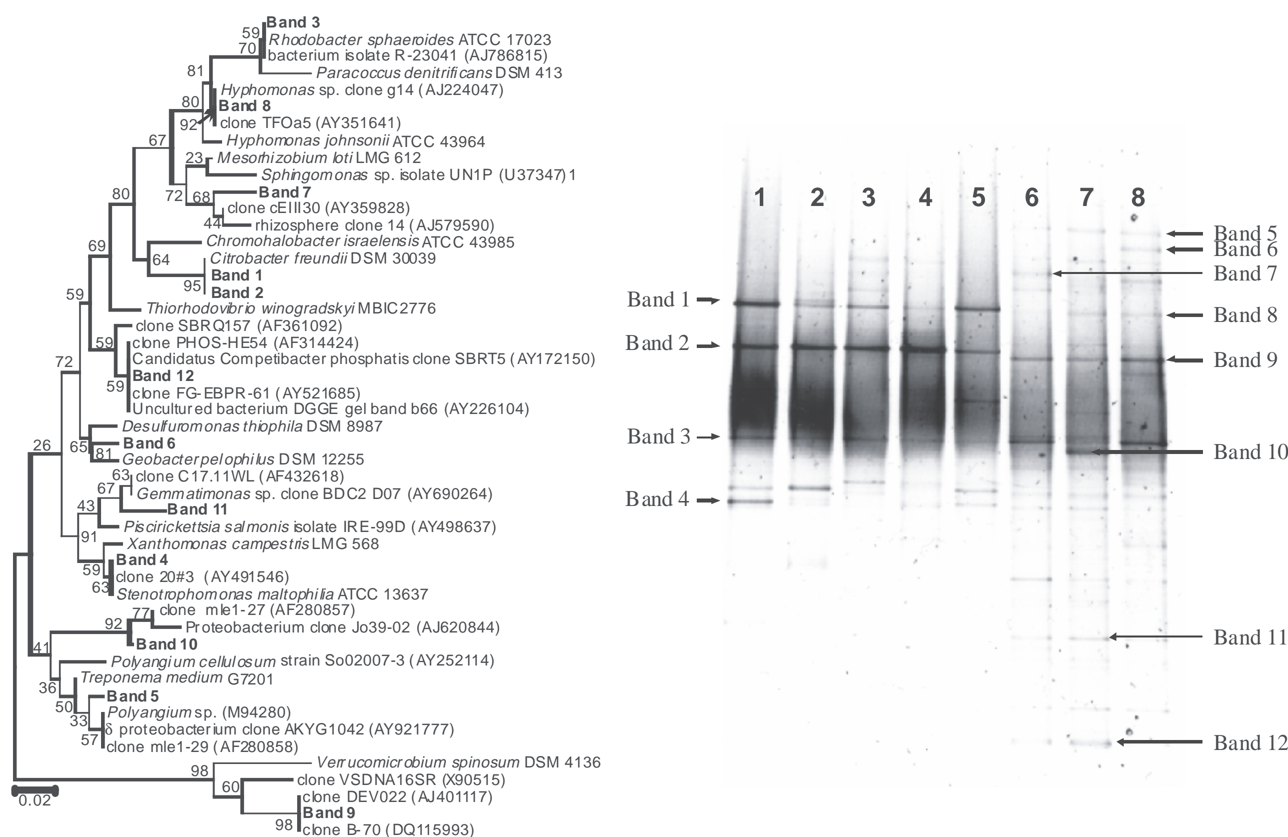


Figure 2.2. Phylogenetic tree of the partial 16S rDNA gene sequences of the isolates obtained from the bands that were excised from the corresponding DGGE. The scale bar represents 0.02 fixed mutation per site. Bootstrap values were derived from 1,000 analyses. The DNA sequences were aligned by using CLUSTAL_X and the tree was constructed using MEGA ver. 3 by the NJ method from a similarity matrix of pairwise comparisons made using the Kimura-2-parameter algorithm. On the right of the figure are the corresponding DGGE profiles of the seed and biomass samples taken during the trial. Lanes: 1, seed sludge; 2–8, samples taken from the oxic phase of the reactor after Periods I, II, III, IV, V, VI and VII, respectively (see Table 2.1 for details).

following Periods V, VI and VII (Fig. 2.2, Lanes 6–8) are quite notable. Only one of the dominant bands that was present in the previous five profiles is present in the remaining three (Band 3), while a number of other bands have appeared in these profiles. The more dominant bands that were present up until this time disappeared and the final three biomass samples also exhibit much greater diversity, with an average of 18 bands in each profile, than the previous five, which contain an average of eight bands in each profile. The analysis of the diversity indices shows that prior to this point the average diversity was significantly lower than after Period IV; average values of Shannon–Weaver (H'), Fisher's alpha (α) and Simpson's (D) indices for the Periods I–IV were 0.61 ± 0.057, 0.94 ± 0.23 and 0.29 ± 0.047, respectively, while for Periods V–VII the average values were 0.91 ± 0.16, 2.71 ± 0.73 and 0.18 ± 0.071, respectively. These values were significantly different for the two periods tested, and all showed P values <0.03. The percentage similarity between the DGGE profiles for Periods IV and V was 50%, which contrasts with percentage similarity prior to this which was 73% (between Periods III and IV) and after this which was 71% (between Periods V and VI). These results indicate a significant change in the community structure. This sudden change in reactor population coincides with a marked deterioration in the performance of the reactor with respect to P removal efficiency. As previously mentioned, the only parameter that was not controlled was the influent waste water and the changes in populations are most likely due to a change in the waste-water characteristics.

A number of bands extracted from the gel were sequenced, phylogenetically analysed and compared to previously published sequences in the EMBL database. The most dominant bands in the first five samples, Bands 1 and 2 (Fig. 2.2), were found to be affiliated with members of the *Enterobacteriaceae* and had 100% identity (290/290 nt) with the following bacteria *Citrobacter freundii*, *Enterobacter amnigenus*, *Kluyvera cryocrescens*, *Raoultella terrigena*, *Klebsiella* sp. TNT1 and *Pantoea agglomerans*. These bands had completely disappeared from the subsequent three samples. The last three samples also showed the appearance of Band 12 (99% identity; 335/338), which was found to be similar to '*Candidatus* Competibacter phosphatis', which are known as glycogen-accumulating organisms (GAOs), a further indication that conditions within the reactor had changed from those favouring PAOs to those favouring GAOs.

2.2.3 Analysis of the 16S rRNA gene clone library

In order to obtain a deeper insight into the changes in the microbial populations between Periods IV and V, samples from Days 73 and 85 were used to construct 16S rRNA gene clone libraries. These were the same samples from which DNA was extracted and used to generate the DGGE profiles shown in Fig. 2.2. The libraries constructed for these samples contained approximately 190 clones from each sample, which were screened using restriction fragment length polymorphism (RFLP) analysis. A total of 21 restriction profiles or OTUs were obtained and the distribution of these OTUs between the two samples is shown in Fig. 2.3 while the phylogenetic analysis is shown in Fig. 2.4. The most notable observation was the

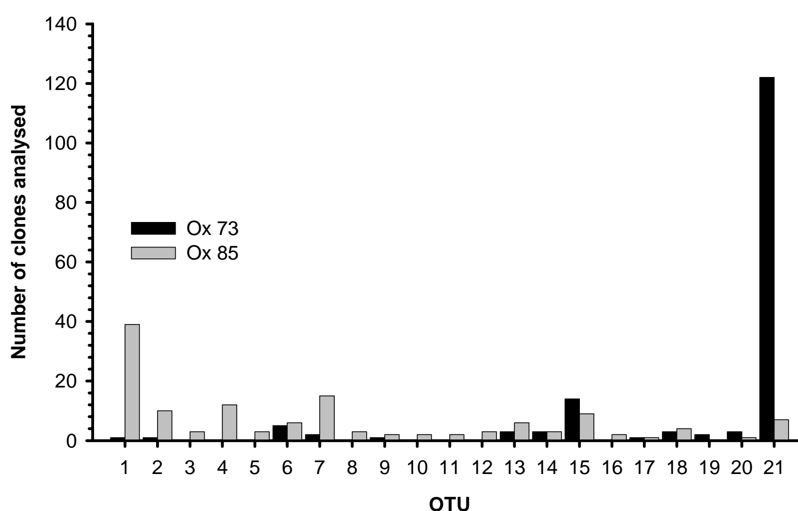


Figure 2.3. Distribution of OTUs within samples taken on Days 73 and 85.

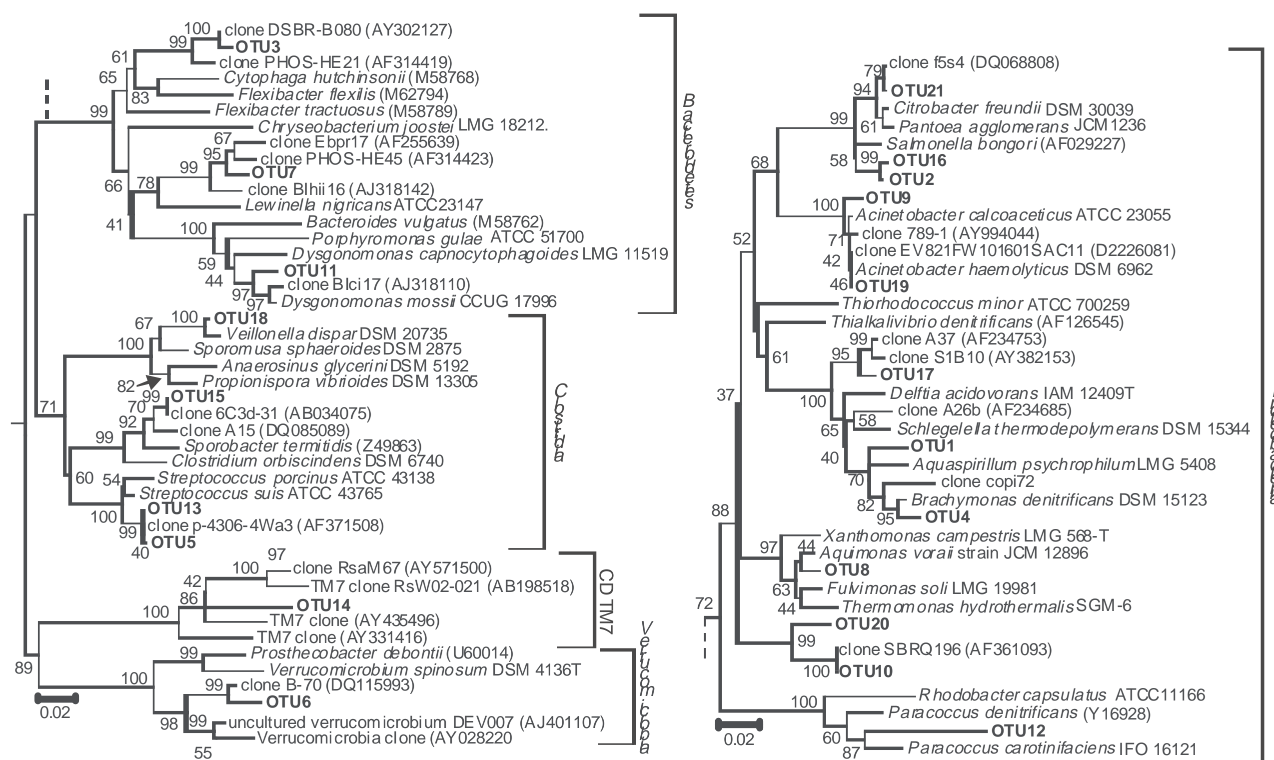


Figure 2.4. Phylogenetic tree of the partial 16S rRNA gene sequences retrieved from the two clone libraries generated from DNA extracted from Samples 73 and 85.

dominance of OTU 21 in Sample 73 when compared to Sample 85. This OTU, following sequence analysis, was found to be similar to *Citrobacter freundii* CDC 621-64 (100% identity; 659/659), which clusters in the *Enterobacteriaceae* family. OTU 9 was found to be closely related to *Acinetobacter* spp., which exhibits the characteristics of PAOs when grown in pure culture. However, this OTU did not comprise a significant part of the total population of either sample (<1.5% of the total number of clones analysed). A number of OTUs identified were also similar to sequences previously isolated from nutrient removal systems (OTUs 3, 7, 10 and 20). The occurrence of most of these OTUs increased in Sample 85, particularly OTU 7, which comprised 8% of the total number of clones analysed for the Day 85 sample, as opposed to 1% for the Day 73 sample. The most dominant OTU in Sample 85 is OTU 1, which was found to be similar to a previously uncultured β-proteobacterium clone DR-29 (AY945920) and which has been implicated in denitrification. A further observation was the diversity exhibited in Sample 85 as compared to Sample 73. A total of 20 of the 21 OTUs were found in Sample 85, as opposed to 14 found in Sample 73, supporting the DGGE finding that Sample 85 was more diverse than Sample 73.

2.2.4 ^{1}H NMR spectroscopy of waste water

^{1}H NMR spectra of the Day 73 (A), and Day 85 (B) samples are shown in Fig. 2.5. Resonance assignments were made according to the literature (Nicholson *et al.*, 1995) and confirmed by 2D COSY and TOCSY experiments. Clear differences between the two samples were observed. The spectra of the Day 73 sample were dominated by a number of VFAs and other metabolites, including butyrate, proprionate, ethanol, pentanic acid, acetate, ρ-cresol and phenylacetate, whereas the spectra of the Day 85 sample contained glucose, ethanol and a range of amino acids including leucine, isoleucine, valine, threonine, alanine, lysine, arginine, glutamate, taurine, glycine, tyrosine and phenylalanine.

2.3 Discussion

The biological removal of P from waste water is unpredictable in its reliability. The P removal efficiency of the A^2/O reactor used in the present study had continued to improve from the start of the trial until Day 85 when it declined rapidly, the reasons for which were unclear. The decrease in the SRT from 17 to 12 days on Day 58 did not adversely affect the P removal and the period immediately

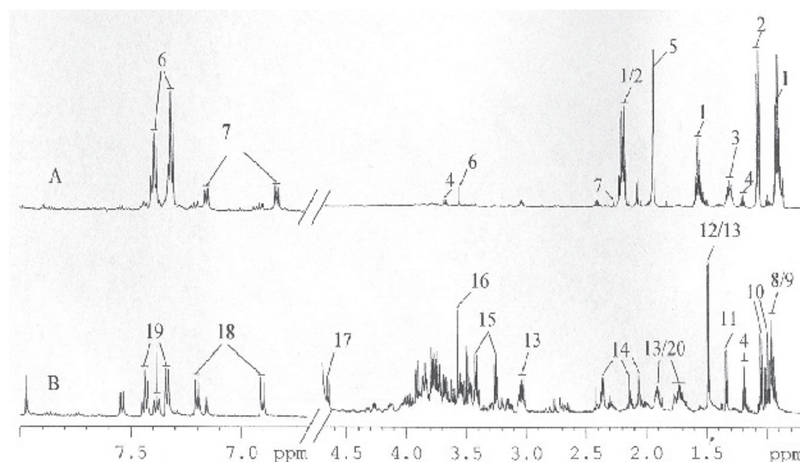


Figure 2.5. 600 MHz ^{1}H NMR spectra of waste water at Day 73 (A) and Day 85 (B). (Key: 1: butyrate; 2: proprionate; 3: pentanic acid; 4: ethanol; 5: acetate; 6: phenylacetate; 7: ρ-cresol; 8: leucine; 9: isoleucine; 10: valine; 11: threonine; 12: alanine; 13: lysine; 14: glutamate; 15: taurine; 16: glycine; 17: glucose; 18: tyrosine; 19: phenylalanine; 20: arginine.)

following the SRT change saw the best P removal efficiency of the entire trial. Other studies have also found reactors to maintain good P removal when operated at an SRT of approximately 10 days. Chuang *et al.* (1997) observed anoxic and aerobic P uptake when operating at an SRT of 10 days. In addition, two other groups (Chang *et al.*, 1996; Choi *et al.*, 1996) have both reported higher P removal at a 10-day SRT, than at either shorter or longer SRTs. However, operating reactors at short SRTs may result in wash-out of slow-growing micro-organisms, such as nitrifying bacteria. Eschenhagen *et al.* (2003) have previously reported that an anaerobic/oxic (A/O) reactor operating at an SRT of 4.4 days removed only 25% of the influent N, while a Phoredox system operating at an SRT of 18 days and treating the same waste water resulted in removal of 80% of the influent N. A low nitrification rate was not evident in the present study since complete N removal was achieved at all stages during the trial. Increasing DO concentrations from 2 to 4 mg/l has been found to improve nitrification at shorter SRTs (Rothman, 1998); thus the DO in the aerobic phase of the A^2/O operated in the present study was maintained higher than 4 mg/l at all times (data not shown).

It is unlikely that the decrease in SRT was the cause of the deterioration in P removal. Other factors such as pH (Schuler and Jenkins 2002; Serafim *et al.*, 2002) and temperature (Converti *et al.*, 1995; Brdjanovic *et al.*, 1998) have been reported to affect biological P removal. However, in the present study, the pH and operational temperature were relatively constant throughout, and would not be deemed a significant factor in the decline in P removal efficiency. Most other parameters, including sludge volume index (SVI) and MLVSS were stable during the trial (data not shown). The only parameter that was not controlled was the influent waste water and, therefore, a change in the influent waste-water characteristics was probably the most likely cause for the declining reactor performance.

It is generally believed that the substrate used by PAOs must be present in waste water either as VFAs or as a compound that can be easily converted to VFAs (Carlsson *et al.*, 1996). The type of VFA is also an important factor. Some studies have found propionate to enhance EBPR. Chen *et al.* (2004) found that higher propionic acid, while detrimental in the short term, led to superior EBPR in long-term cultivation. Oehmen *et al.* (2005) have also previously reported that propionate can promote the growth of PAOs over GAOs, while acetate had the opposite effect. Rustrian *et al.* (1996) showed that both acetate and butyrate are good C sources for P removal while propionate did not promote good P removal. It is suspected that a change in the VFA profile of the waste water during Period V, or the appearance of a compound not easily converted to VFAs, is possibly responsible for the decline in P removal that was observed in this study. ^{1}H NMR analysis of the waste water taken on Days 73 and 85 showed a marked difference in the waste-water composition between these

two samples. The waste water sampled on Day 73 was found to be rich in propionate, butyrate and acetate, the VFAs of which have all been shown to be essential substrates for PAOs, along with other metabolites (pentanic acid, phenylacetate and ρ-cresol). In contrast, the main constituents of the Day 85 waste water were amino acids, which have been reported to be detrimental to the P removal process. The decline in P removal coincided with this change in influent characteristics, showing that the availability of VFAs was essential to the P removal process in the reactor. It is believed that the VFAs measured in the waste water are a reasonable surrogate indicator of influent quality since phylogenetic analysis of the bacterial communities in these two samples did not show any significant changes in the potential VFA producers at Days 73 and 85, i.e. the *Clostridia*.

Both DGGE and cloning results showed the disappearance of the most dominant *Enterobacteriaceae*-like organisms from the system between Days 73 and 85 of the trial, which included denitrifiers, heavy metal accumulators and N fixers. While it is unclear what function these organisms had within the reactor, they may have played an important role in the P removal process, either as PAOs themselves or, more likely, in providing the necessary substrates (VFAs) for P removal. This indicates that even though an organism may not be directly involved in P removal, its presence may be just as vital to the P removal process as the PAOs themselves and highlights the necessity for the combined actions of different microbial groups in achieving complete P removal. Many of the other band and clone sequences retrieved were unclassified and their functions within the reactor remain unknown. *Acinetobacter*-like organisms were also detected in the reactor, the role of which has been the subject of much discussion. While these organisms exhibit the characteristics of PAOs in pure culture, their significance in EBPR sludge is disputed. The use of molecular techniques has revealed that other organisms, such as *Rhodocyclus* (Bond *et al*., 1995; Zilles *et al*., 2002) and *Tetrasphera* (Danalewich *et al.,* 1998) play a more significant role in EBPR. However, these organisms were not detected in this system by either of the methods used. What is notable from the DGGE analysis is the appearance of an organism similar to '*Candidatus* Competibacter phosphatis', a known GAO which has been found in both laboratory-scale and full-scale waste-water treatment systems (Crocetti *et al*., 2002). Although it is not a significant band within the overall DGGE profile, it does indicate that conditions within the reactor had changed from those favouring PAOs to those favouring GAOs.

2.4 Conclusions

Thus, using culture-independent approaches (both ^{1}H NMR and molecular) this study showed that in laboratory-scale EPBR processes significant changes in the community structures and its metabolome can be linked to a failure to efficiently remove PO_4. It appears likely that the key factor influencing this decrease in PO_4 removal efficiency was more than likely due to changes in the influent composition and in particular the quality and quantity of short-chain fatty acids (SCFAs). Further work will need to be undertaken in order to confirm this conclusion. It appears that the control of influent quality may be a key factor to aid in the successful removal of PO_4 by EPBR systems and failure to ensure sufficient levels of SCFAs may be a contributory factor to their malfunction.

3 Project 2: Investigate Acid-Stimulated Phosphate Removal and Phosphate Accumulation by Activated Sludges from Food-Industry Waste Streams

3.1 Project Overview

Previous work by McGrath *et al*. (2001) at Queen's University Belfast had reported an acid-stimulated luxury PO_4 uptake by environmental micro-organisms. They demonstrated that bacterial cultures displayed an increase of between 50% and 143% in levels of PO_4 uptake when the pH of the growth medium was reduced from 7.5 to 5.5. Enhanced PO_4 removal by these cultures was accompanied by increases of between 2- and 10.5-fold in their polyphosphate content. They proposed that this previously unrecognised phenomenon may have application in novel technologies for nutrient removal from waste waters.

Further work by this group has aimed at developing a prototype for the development of alternative biological and chemical options for PO_4 removal from waste waters, using this acid-shock based approach. They have employed a 2000-l activated sludge pilot plant which has been constructed at a municipal sewage treatment works. When operated as a single-stage reactor, they have reported that this system can remove more than 60% of influent PO_4 from primary settled sewage at a pH of 6.0, as opposed to approximately 30% at the typical operational pH for the works of 7.0–7.3, yet without any deleterious effect on other treatment parameters (Mullan *et al*., 2006).

Thus, this project set out to establish whether or not this phenomenon of acid-stimulated PO_4 removal and poly-P accumulation could be used in a novel process to treat high-PO_4 waste streams from the food processing industry.

3.2 Results and Discussion

Initially the design criteria obtained from the Queen's research group were employed and a small laboratory-scale system was commissioned to investigate the acid shock phenomenon with food-industry waste streams. However, some unforeseen design issues arose as the reactor design required modification, due to the differences between municipal and food-industry waste water. While treating food-industry waste water, reactor issues arose associated with elevated N concentration in the waste water, such as poor settlement of the sludge primarily due to denitrification in the clarifier. This highlighted the need for a system with a dedicated anoxic zone to negate this effect.

Subsequently, a reconfigured, small laboratory-scale, waste-water treatment system was set up (Fig. 3.1). This new reactor was calibrated, maintained and examined under neutral pH conditions (pH 7.5). From previous trials carried out in the laboratory, it was deemed appropriate to initially feed the reactor with a synthetic waste water rather than the desired food industry waste streams. This would facilitate the stabilisation of the system and also allow for greater control over the nutrient removal capacity of the reactor. However, during operation of this new system, design faults were discovered, with recycling of activated sludge to the reactor not functioning as required. Thus sludge was collecting in the clarifier and as a result was reverting to an anaerobic state. In order to overcome this problem, a new clarifier with an attached stirring mechanism was integrated into the system. As a direct result, recycling rates were improved satisfactorily. At this point, the reactor was removing approximately 30% P and N removal was continuously improving. However, after a brief period of time sludge recycling problems were again encountered and it was decided to reconfigure the entire system to an SBR design rather than to further change the clarifier design. Changing to such a reactor design would eliminate the need for an additional clarifier and therefore sludge recycling would cease to be a problem.

An SBR design was then implemented (Fig. 3.2), with the system being initially maintained and studied under neutral pH conditions (pH 7.5) and following increases to the DO content of between 2 and 4 mg/l, around 30–50% P and approximately 90% of N present in the synthetic waste water were converted to nitrate (NO_3). To further improve the conversion of NO_3 to N gas, an anoxic zone was subsequently incorporated into the system at a ratio

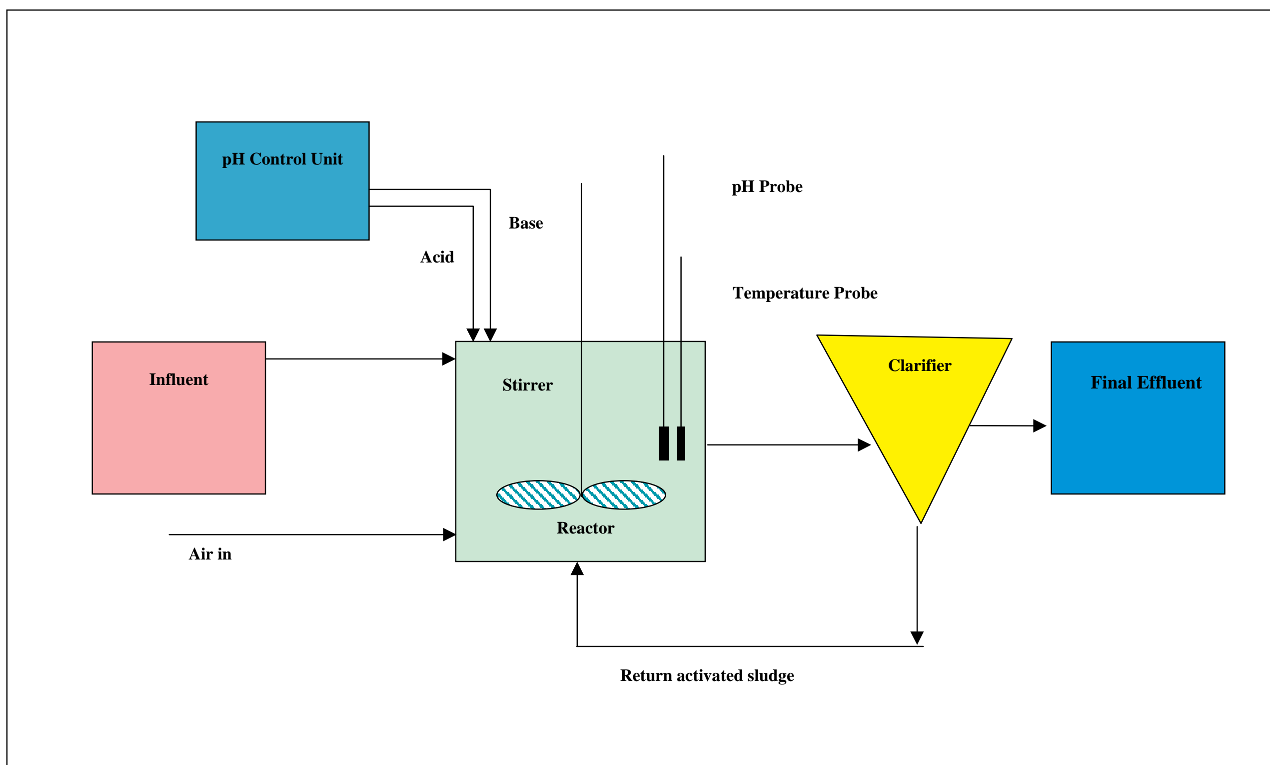


Figure 3.1. Schematic of initial reactor design.

Figure 3.2. Sequencing batch reactor set-up for acid-shock work.

of 4:1 aerobic conditions to anoxic conditions. Under these operating conditions, the reactor removed around 30% of the P, with approximately 90% of the ammonia (NH_3) being removed while being fed the synthetic waste water stream.

Upon continued operation of the SBR it became apparent that the efficacy for BNR diminished markedly with sludge age, with significant quantities of ciliates being observed such as for example ‘Bristle worms’, which are typically associated with sludge ageing and increasing NO_3 accumulation. This problem was overcome by decommissioning and cleaning the system.

The SBR continued to be operated under oxic–anoxic cycling of activated sludge performing BNR on synthetic waste-water influent. The operating conditions are outlined in Table 3.1.

Under the above operating conditions, chemical analysis of influent and effluent samples was performed to assess the effects of pH change on BNR (Fig. 3.3). Routine microscopic analysis was also performed on the sludge to monitor overall sludge ‘health’.

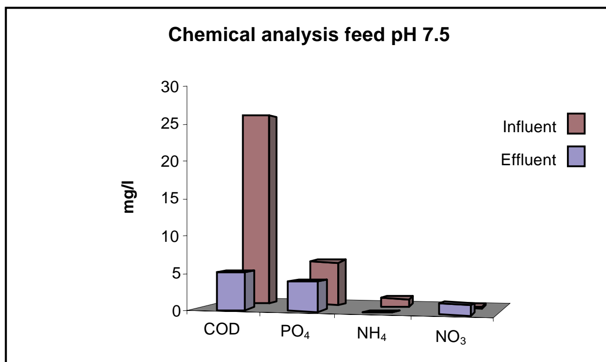
The average percentage nutrient reduction did not vary significantly and can be grossly represented in Table 3.2.

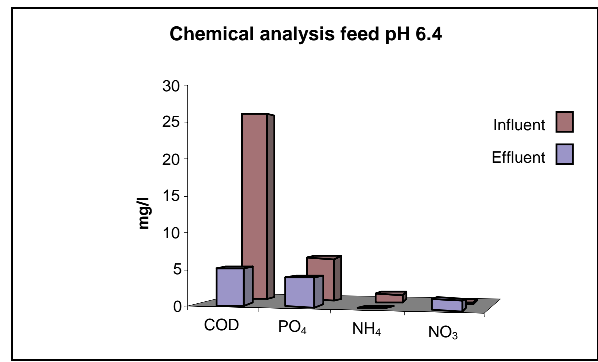
Table 3.1. Reactor cycle time: 120 min.

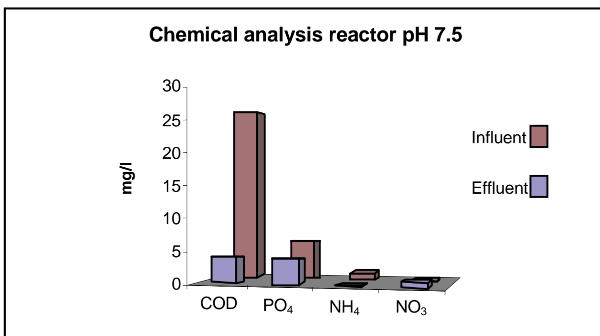
Time (min)		Influent feed	Aeration	Stirring	Effluent draw
0	**Oxic Zone**[1]	ON	ON	ON	
3		OFF			
84	**Anoxic Zone**[2]		OFF		
105				OFF	
112					ON
115					OFF

[1]Oxic Zone: 84 min (C utilisation, PO_4 removal by acid shock, nitrification).

[2]Anoxic Zone: 21 min (denitrification).







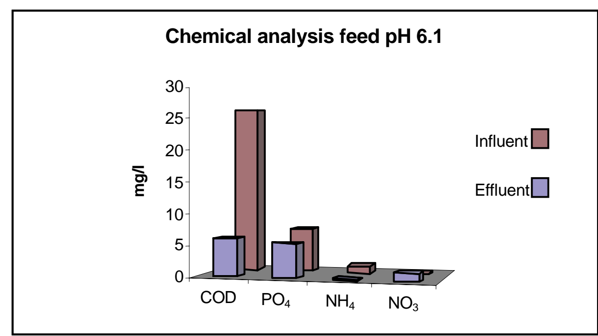


Figure 3.3. The effects of pH change on biological nutrient removal.

Table 3.2. Average percentage nutrient reduction.

Nutrient	Percentage reduction
COD	96
PO_4	24
NH_4	94
NO_3	87

To generate samples which could be used for molecular analysis work to monitor the predominant PAOs operating within this system, the operation of the rigs was modified to reflect dairy-industry waste-water composition. Fresh sludge was obtained from the settling tank of Kerry Ingredients, dewatered and used to achieve reactor-mixed liquor suspended solid densities of 4 to ~6.

A synthetic medium was supplied to the reactors, based on seasonal nutrient fluctuations in the Kerry Ingredients waste water:

Low season (winter): COD: 2,200 mg/l, PO_4: 32 mg/l, NO_3/NH_4^+: 33 mg

High season (spring/summer): COD: 2,200 mg/l, PO_4: 35 mg/l, NO_3/NH_4^+: 65 mg

Synthetic media 1: COD: 2,000 mg/l (lactose), PO_4: 30 mg/l, NO_3/NH_4^+: 25 mg/l.

The reactor operational cycle was two hourly (see Fig. 3.4).

It was observed during continuous culturing with this medium over an initial 2-month trial period that the sludge critical stability was easily exceeded due to the high COD-

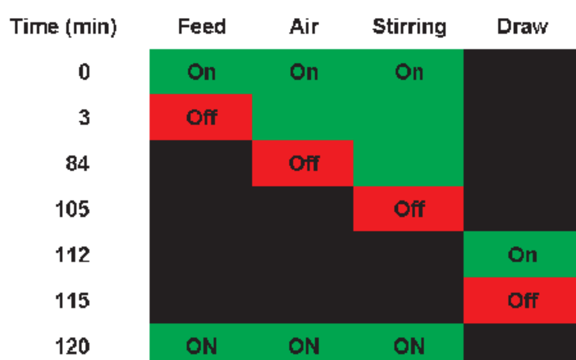


Figure 3.4. Reactor operational cycle. (Despite the transition away from anaerobic cycling as an inducer of EBPR, the anoxic phase was maintained to induce nitrification/denitrification).

induced biomass accumulation. Phosphate and nitrate profiles were inconsistent with EBPR, with the low levels of reduction more in keeping with incorporation into biomass substructures (i.e. cell wall components, proteins, nucleic acids, etc.). Less than 40% utilisation of COD was being achieved, with calculations being compounded by microbial contamination of the feed reservoirs. This problem was addressed by reducing COD in the synthetic medium by 50% to 1,000 mg/l, while maintaining the original inorganic nutrient concentrations. Furthermore, the pH of the reservoir was reduced to investigate the effects on contamination and acid-shock-induced P uptake in the reactor, compared with the control reactor where pH was not modified. A 12-week trial was undertaken with the reactors operating under these conditions, revealing the following nutrient utilisation profiles shown in Fig. 3.5.

The data from Reactors 1 and 2 indicate the importance of achieving microbial control in feed reservoirs via reduced pH. As Reactor 2 shows, the influent nutrient concentrations were severely affected by contaminating

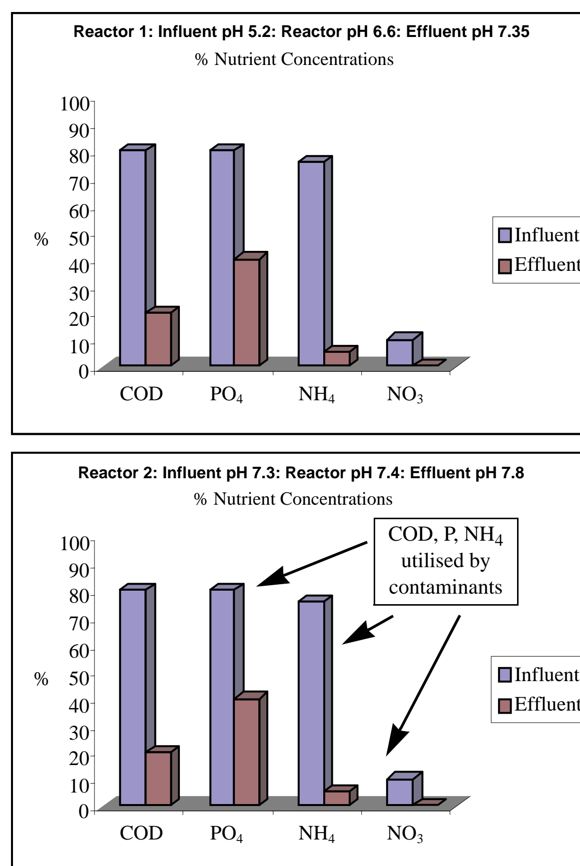


Figure 3.5. Nutrient utilisation profiles.

lactose utilisers, to the extent that insufficient COD was entering the reactor to maintain the sludge sufficiently to promote effective PO_4 removal. Furthermore, the inorganic nutrient concentrations entering the reactor were artificially reduced, which is an unlikely event in a real-time waste-water treatment scenario. Interestingly, acidification of the influent to pH 5.2 had the effect of achieving a low reactor pH with concomitant 50% PO_4 removal. This should be further investigated as regards to acidification of industrial-scale waste water prior to treatment, which would be particularly useful in the spring/summer peak period where recorded fluctuations in NH_4 ion concentrations have serious impacts on influent pH fluctuations. Future goals should also include investigating the potential to acclimatise the sludge with increasing COD concentrations. It is likely, however, that the treatment of industrial waste-water COD will require the incorporation of a pre-EBPR reactor, sludge exposure step to mimic, in a controlled fashion, the effects of reservoir contamination reported here.

3.3 Molecular Analysis

Molecular analysis was then performed on the microbial populations present within the activated sludge samples from the SBR. Initially a 16S rRNA clonal library was constructed based on cDNA generated by reverse transcription of total RNA isolated from the sludge samples, followed by RFLP analysis, using three separate restriction enzymes. The RFLP banding patterns generated were manually screened to identify potential homology groupings, which would be indicative of the presence of dominating species within the cloned 16S fragments, and ultimately the activated sludge. Representative clones were then sequenced to facilitate bioinformatic analysis and species-level identification.

Bioinformatic-based analysis was performed on three separate groups, using the (RDP)II database, a database dealing solely with 16S rRNA genes. Using the associated sequence match algorithm, it was possible to reliably identify many of the cloned 16S genes at the genus level. Group B, representing the dominant RFLP group (28%) were found to belong to the genus *Verrucomicrobium,* while comparison of the Group C representative (10%) identified the presence of members of the β-*Proteobacteria*, genus *Rubrivivax*. The remaining Group D 16S RFLP profiles (8%) were identified as members of the *Proteobacteria,* subclass δ-*Proteobacteria*. The significant number of unique banding patterns, which could not be grouped appropriately for sequencing, indicates a broad microbial diversity in the activated sludge community and the possibility cannot be excluded that multiple bacterial species within these populations may contribute to enhanced nutrient removal by the sludge.

While it is clear that the genus *Verrucomicrobium*, which represents 28% of the cloned genes analysed, was identified as an abundant component of global soil microbial communities, of greater significance, however, is the presence of *Verrucomicrobia* in the nitrifying–denitrifying activated sludge of an industrial waste-water treatment plant (Juretschko *et al*., 2002), where they represented approximately 5% of the microbial populations identified. This finding is consistent with this study's chemical analysis of waste-water influent and effluent, where BNR by the activated sludge accounted for 99% and 87% removal of NH_3 and nitrates, respectively, suggesting the presence of nitrifying–denitrifying bacterial genera within the sludge under current operating conditions.

The identification of the genus *Rubrivivax* (representing 10% of the cloned 16S rRNA genes) is significant also as this group was previously classified in the genus *Rhodocyclus*, which has recently been reported as having a crucial role in PO_4 removal in several activated sludge environments (Seviour *et al*., 2003). The lower percentage abundance of this species in comparison to the *Verrucomicrobia* is most likely due to the reactor conditions. Indeed, this study is likely to generate a unique microbial ecology profile as it utilises oxic and anoxic cycles in conjunction with acid pH stress to induce P removal, while standard systems utilise anaerobic–aerobic cycling. Furthermore, P removal has been suboptimal in the past (27% removal) as pH within the reactor fluctuates rapidly with C utilisation and nitrification of ammonium ion – resulting in rapid increases from pH 6.1 to 8.4. Thus, there would appear to be a selective advantage for nitrifying–denitrifying micro-organisms dominating the sludge, which this study's molecular data support.

In an attempt to confirm these relative abundances, and to further investigate the influence of varied growth conditions on the overall microbial ecology of these systems under study, a fluorescent *in situ* hybridisation (FISH)-based approach was initiated to provide visual confirmation of the molecular data within the sludge. FISH

involves the design of oligonucleotides with a 5' fluorescent label which are targeted against species-specific 16S rRNA molecules. Visualisation of hybridised probes is typically achieved via confocal microscopy, although the flocculent nature of activated sludge sometimes requires a three-dimensional analysis approach, provided by laser scanning microscopy. However, problems were encountered in using FISH to specifically identify PAOs in the sludge samples under study, particularly with non-specific binding using these *in situ* probes. The reason for this is not readily apparent. In a parallel approach staining with 4'-6'-diamino-2-phenylindole (DAPI) was employed to allow a distinction to be made between PAOs and non-PAOs. DAPI has previously been successfully employed to monitor PAOs in activated sludge samples by other research groups (Liu *et al.*, 2006). In this way it was hoped to visualise PAO micro-organisms containing intracellular polyphosphate granules and those without. Initial results obtained appeared to indicate that staining with DAPI would allow a distinction to be made between micro-organisms with intracellular polyphosphate granules and those without. However, the initial results obtained were not routinely repeatable and despite repeated attempts a high level of non-specific binding was observed and despite further attempts to optimise the DAPI staining protocol, this difficulty remained unresolved. Again, as with the problems encountered using the Cy3 and Cy5 *in situ* probes, the reason behind the problems encountered are not readily apparent.

4 Project 3: Investigate the Feasibility of Using Sludge Generated in a Biological Phosphate Removal Plant as a Feedstock for Compost

4.1 Project Overview

Given that composting is a treatment option that is increasing in Ireland, there is a need to ensure that both the product (compost) and the process (composting) are of a suitable standard and quality. The first task in this project was to develop a compost quality testing regime, whereby compost samples could be monitored for four important criteria: (1) maturity, (2) foreign matter, (3) trace elements, and (4) pathogens. The second task was to design and construct a pilot-scale composting unit.

4.2 Introduction

With regard to the use of tests to assess compost quality, it is essential to achieve and maintain a high standard of quality, consistent with requirements in all aspects of work carried out within the laboratory, if technically valid results are to be generated. To ensure the accuracy of the analytical testing all compost quality testing methods were validated prior to implementing these techniques as standard test methods in the laboratory. Table 4.1 shows the tests involved. The first stage of this project involved the development and validation of these tests.

In order to facilitate the validation of the various methods involved in compost quality testing, two compost samples were used. The first sample was a commercially available product that, presumably, had completed and passed a set of similar testing procedures. This sample was produced by windrow composting and is referred to as Sample 1 for the duration of the validation procedures. The second sample was a recently produced compost sample, which was not on the market and had not previously undergone any quality assessment test. It was produced by vermicomposting and was labelled Sample 2 for validation purposes. The composting process can be characterised by a high degree of interactions of several biochemical and physical factors in a heterogeneous matrix of gas, liquid and solid phases, which fluctuate markedly over time (Sole-Mauri *et al.*, 2007); thus, this study set out to monitor changes in temperature, moisture content, C and N ratios, and overall respiration rates in experimental compost units.

Table 4.1. The compost quality tests.

Test	Compost criteria
C/N ratio	Maturity
O_2 uptake	Maturity
Cress test	Maturity
Sieve test	Foreign matter
Heavy metal analysis	Trace element levels
Recovery of *Salmonella* sp. using the MPN method	Pathogen levels
Recovery of faecal coliforms using the MPN method	Pathogen levels

4.3 Materials and Methods

4.3.1 Bacterial strains

The bacterial strains used, namely *Salmonella* species and *Escherichia coli*, were obtained from the culture stocks at the Microbiology Department at University College Cork. Stock cultures were maintained in tryptone soy broth medium and stored in stock tubes at 20°C.

4.3.2 Growth media

The types of growth media employed are given in Table 4.2.

Table 4.2. Growth media used.

Media used	Company
Agar	
Triple Sugar Iron Agar Slants	Merck (2002)
***Salmonella–Shigella* Agar**	Merck (2002)
Brilliant Green Agar	Oxoid (2002)
Modified Brilliant Green Agar	Oxoid (2002)
Violet Red Bile Agar (VRBA)	Oxoid (2002)
Broth	
A-1 Medium	DIFCO (2002)
Rappaport–Vassiliadis Broth	Oxoid (2002)
Tryptone Soy Broth	DIFCO (2002)

4.3.3 *Determination of the water content*

A representative sample was weighed in for the determination of the water content, loaded into suitable vessels and dried at 105°C up to constant weight (24 h). The sample was placed in a desiccator immediately after removal from the oven to prevent uptake of moisture. It was then allowed to cool to room temperature and weighed with suitable scales directly after removal from the desiccator. Since the water content is related to the fresh mass, the result was recorded in percentage dry substance (%DS), accurate to one place of decimal. For the determination of water content, a small representative sample of approximately 10 g was deemed to be sufficient. Two replicates of each sample were used to determine the water content and the average value was taken to be the estimated percentage water content.

The following formulae were used for the determination of the water content and dry substance content of the sample:

$$WG = ((M_{moist} - M_{dry}) \div (M_{moist} - M_{tara})) \times 100\ (\%)$$

$$DS = ((M_{dry} - M_{tara}) \div (M_{moist} - M_{tara})) \times 100\ (\%)$$

where WG is the water content (%DS), DS is the dry substance (%DS), M_{tara} is the mass of the empty plate (g), M_{moist} is the mass of the moist sample + M_{tara} (g), and M_{dry} is the mass of the dried sample + M_{tara} (g).

4.3.4 *Determination of the pH value*

The pH value was determined electrometrically using a suspension of the fresh compost substance in 200 ml of 0.01 molar $CaCl_2$ solution (1:10 ratio), determined after 1 h with a calibrated pH-measuring device. The temperature of the suspension was taken into account during the measurement in accordance with the type of measuring device. The results of the pH determination were recorded to one decimal point.

4.3.5 *Determination of conductivity*

The determination of the conductivity of the sample occurred after extraction with distilled water (1:10). The sample was first sieved to <10 mm. Then 20 g of sample were added to 200 ml distilled water and the mixture was continuously mixed for 1 h. The conductivity of the sample was then determined using a conductivity measuring device (FCQAO, 1994).

4.3.6 *Determination of the content of seeds present and capable of germinating in the potting soil*

This procedure was carried out to investigate the following:

- If the potting soil contained any seeds, which, given suitable growth conditions, could hamper the growth of plants during the toxicity trial.
- To determine if varying amounts of the potting soil had an effect on the germination or growth of the plants.
- If the potting soil could indeed support the growth of cress seeds, which are used in the phytotoxicity test.

The potting soil was sieved to <10 mm before use. The amount of moisture present in the fresh sample was determined and adjusted accordingly to equate to 70% moisture level. Two replicates of each treatment were evaluated along with two control pots for each treatment (Table 4.3).

A known amount of seeds were planted in each of the test pots containing the potting soil. The control pots, also containing the potting soil, were left unsown in order to determine if any seeds were naturally present in the soil. The pots were placed in a growth chamber for a period of 7 days at 18°C, while being exposed to fluorescent light for 16 h daily. The moisture levels of the pots were checked daily and changed accordingly if needed throughout the duration of the trial.

The pots were removed from the growth chamber on Day 7. The control pots were examined for evidence of any plant growth. The test pots were examined to facilitate determination of the effects of varying amounts of soil and also to ensure that the soil could support the growth of

Table 4.3. Amount of soil used in the determination and evaluation of the content of seeds present and capable of germinating in potting soil.

Treatment	% potting soil in test pots	% potting soil in corresponding control pots
1	100	100
2	90	90
3	80	80
4	70	70
5	60	60
6	50	50

plant life. The plants were removed from the test pots, weighed and their weights recorded. They were then dried at 105°C in a drying chamber overnight. Their dry weights were obtained and compared to investigate the effects, if any, of using varying amounts of soil.

4.3.7 Phytotoxicity

Planting garden cress (*Lepidium sativum*) seeds in different potting soil/compost ratios was used to determine the phytotoxicity of compost samples. The potting soil used was topsoil. Two replicates per potting soil/compost ratio were prepared by filling pots (diameter of 7.5 cm, height 8 cm) with the required compost/potting soil mixture and with enough deionised water to promote germination. In addition, two controls each of potting soil without compost and compost without potting soil were held under the same conditions.

To estimate the total volume of the containers that were used in the experiment, a single container was filled to an acceptable level (i.e. approximate level to which all containers were filled throughout the experiment) with potting soil. The contents of the container (i.e. potting soil) were then transferred to a graduated cylinder to calculate the total volume of the container in millilitres. From this, the correct mixture of potting soil and compost that was used to evaluate the growth rate and germination rate of cress seeds at different potting soil/compost ratios was calculated. The potting soil/compost ratios, which were analysed, are shown in Table 4.4.

A known number of cress seeds were placed in the pots and covered lightly with the desired potting medium. Moisture levels were brought up to 70% moisture. The pots were placed in large holding containers and held at 12°C with a 16-h light and 8-h dark photoperiod for 7 days. Pots were monitored and watered with deionised water as needed throughout the trial to maintain the moisture level at 70%.

Table 4.4. The potting soil/compost ratios examined in the phytotoxicity test.

Potting medium	Ratio
Potting soil/compost	50:50
Potting soil/compost	60:40
Potting soil/compost	70:30
Potting soil/compost	80:20
Potting soil/compost	90:10

The number of shoots visible in each pot were counted and recorded on Trial Day 7 to ascertain the germination rate. The plants were cut off exactly between the root and stalk for the harvest. The stalks and roots were then oven dried separately at 105°C overnight. The dry weight was obtained and recorded before placing the samples in an oven at 550°C for 120 min. Growth rate and germination data were based on two observations per mixture and two corresponding controls. An observation was made visually as to whether the compost was an impediment to germination for sensitive plants or not, by using the control pots as a comparison.

4.3.8 Determination of foreign matter and stone content

Foreign matter is all the material that is undesired in the composted material. This is, in principal, all inorganic materials, such as glass, metals, rubber, etc. Foreign matter impairs the optical appearance of compost. The degree of impurity is dependent on the optical conspicuousness of the foreign matter contained in the compost. Only foreign matter with a particle size >2 mm is relevant for the optically effective degree of impurity of compost. Stones are likewise undesirable content matter but not foreign matter. Only stones with a particle size >5 mm are relevant for the evaluation.

The compost material was dried at 105°C overnight. The dried sample was weighed and subsequently passed through a 5-mm sieve and then a 2-mm sieve. The sieved-through fraction <2 mm was discarded. From the sieve fraction >5 mm, stones were picked out and weighed. During the selection, portions of not more than 100 ml were examined each time. The mass of the foreign matter >2 mm was weighed and separately recorded. Foreign matter and stone content relate to the total mass of the dried sample and were recorded as %DS, accurate to two decimal places. The following formulae were used to determine the amount of foreign matter and stone content of the compost sample:

$$Fs = (M_{Fs>2\ mm} \div P_{tot}) \times 100\ (\%)$$

$$St = (M_{St>5\ mm} \div P_{tot}) \times 100\ (\%)$$

where Fs is the foreign matter content (%DS), $M_{Fs>2\ mm}$ is the foreign matter >2 mm (g), St is the stone content (%DS), $M_{St>5\ mm}$ is stones >5 mm (g), and P_{tot} is the total mass of the examined sample before sieving (g).

4.3.9 Determination of the loss on ignition

The loss on ignition supplies a measure for the content of organic substances in composts. Inorganic compounds and water of constitution can also evaporate through this determination method and these are added to the content of organic substances in the evaluation. The fresh sample was placed in a suitable, weighed beaker and dried at 105°C in a drying chamber overnight. After drying, the sample was ground to approximately <0.25 mm and then weighed. The sample was then placed in a suitable oven at 550°C for 120 min. The sample weight after ashing was obtained and recorded (±1 mg). The loss on ignition relates to the dry mass. The results were therefore recorded (%DS), accurate to one decimal place. The following formula was used in the determination of the loss on ignition of the compost sample:

$$GV = ((M_{vdG} - M_{ndG}) \div (M_{vdG} - M_{tara})) \times 100\ (\%)$$

where GV is the loss on ignition (%DS), M_{tara} is the mass of the empty beaker, M_{vdG} is the sample weight + M_{tara} (g) before burning, and M_{ndG} is the sample weight + M_{tara} (g) after burning.

The organic C content was further calculated by multiplication by a factor of 0.58 from the loss on ignition as a measure of the organic substance in composts.

4.3.10 Determination of heavy metals

The dry compost sample was treated with aqua regia for the determination of the total content of heavy metals lead, cadmium, chromium, copper, nickel, mercury and zinc. The elements mentioned were determined in the treatment solution with ICP-MS (induced-coupled plasma mass spectrometry) (Hemschemeier *et al.*, 1991). The fresh sample was dried overnight at 105°C and ground to approximately <0.25 mm. Approximately 0.05 g of the dried sample was placed in a reaction vessel and the exact weight was noted. Hydrochloric acid (3 ml) and nitric acid (1 ml) were added (aqua regia: 3 parts hydrochloric $acid_{conc}$ + 1 part nitric $acid_{conc}$).

The determination of the individual heavy metals in the treatment solution took place using ICP-MS with values given in milligrams/litre. This heavy metal analysis was carried out by the Chemistry Department at UCC.

The levels of heavy metals present were calculated using the following formula:

$$SM = (S_{mal} \times F_{dil} \times V_{mk}) \div E\ (mg/kg)$$

where SM is the heavy metal content (mg/kg DS), S_{mal} is the heavy metal concentration in the treatment solution (mg/l), F_{dil} is the possibly necessary dilution factor, in so far as the standard measurement range was exceeded with individual elements and the treatment solution had to be diluted, V_{mk} is the volume of the treatment solution (volumetric flask) (ml), and E is the weigh-in (g).

4.3.11 Determination of the total N content

The total organically bound N content was recorded through the Kjeldahl treatment of the dry compost substance. The organically bound N is transformed with concentrated sulphuric acid in the presence of salts and suitable catalysts into the NH_3 form. After alkalisation, distillation and collection of the stripped NH_3 in a suitable acid pattern, the NH_3 content can be determined with volumetric analysis.

Kjeldahl analysis was performed based on previously described protocols from the Federal Compost Quality Assurance Organisation (FCQAO, 1994), with samples being dried overnight at 105°C and ground to approximately <0.25 mm. A 1-g quantity of the sample was weighed into a treatment vessel (±mg). The control blank received 1 g of distilled water. Two Kjeldahl tablets were added to each vessel. Concentrated sulphuric acid (10 ml) and hydrogen peroxide (5 ml) were added to each vessel, and three drops of anti-foaming agent were also added to each tube. This treatment took place using a preheated digestion unit at 420°C until the liquid was bright and clear (approximately 90 min). When the sample had cooled to room temperature, the NH_3 was distilled with water steam into a boric acid receiver. The contained NH_3, which was collected in the boric acid receiver, was titrated with 0.14 N hydrochloric acid to facilitate volumetric analysis.

The total N content was then calculated using the following formula:

$$N = (((M_{lp} - M_{lb}) \times M_{wt} \times N_{HCl}) \div W) \times 100\ (\%)$$

where N is the N content (%DS), M_{lp} is the titration value (ml) for the sample treatment, M_{lb} is the titration value (ml) for the blank value, M_{wt} is the molecular weight of N (14.007), N_{HCl} is the normality of hydrochloric acid (0.14), and W is the sample weigh-in (mg).

4.3.12 Determination of the respiration rate

This was determined as previously described (APHA, 1995). Initially electrolyte (25% w/v $CuSO_4 \cdot 5H_2O$) was

added to the manometric cell containing the anode and cathode at a volume of 45 ml. A CO_2 trap (1% NaOH) was added to the appropriate cuvette and mounted on the respirometer at an 18-ml volume. The temperature of the water bath was maintained at 20°C for the duration of the test. An 8-g quantity of fresh sample was mixed with 500 ml of distilled water in a food blender for approximately 1 min. This was then placed in a sterile Duran bottle and the following chemicals were added: 15 ml PO_4 buffer, 5 ml each of CaCl2, $FeCl_3$, $MgSO_4$ and ATU. All chemicals were made up according to the standard methods biological oxygen demand (BOD) test procedures (APHA, 1995). The compost samples were examined by placing 70 ml of the above compost suspension into the appropriate reaction vessels. Two replicates of each sample were examined. The reaction vessels were then mounted on the respirometer and submerged in water, maintained at 20°C.

4.3.13 Determination of the presence of pathogens

The faecal coliforms test was performed using a multiple-tube fermentation procedure. Faecal coliforms can be identified by their ability to ferment lactose, thereby producing gas and acid, at 44.5°C. The procedure used A-1 broth and inverted Durham tubes, with positives being identified by the presence of gas bubbles in the Durham tubes and the discolouration of the media due to acid production.

Once the medium was dispensed into the test tubes, the tubes were capped and sterilised by autoclaving at 121±1°C for 15 min. Fifty grams of the compost sample were added to 450 ml of buffered peptone water (this mixture contained 0.1 g of the original sample). This mixture was placed in a blender and blended at high speed for 2–3 min; 11 ml of this mixture were added to 99 ml of buffered peptone water. Tenfold serial dilutions of this sample were then performed and analysed.

All six series of tubes were incubated at 37°C for approximately 3–4 h and then at 44.5°C for 24 h. Both a positive and a negative control were included in the experimental set-up. The positive control tube was inoculated with 10 ml of a wild-type *E. coli* culture, grown overnight with shaking in tryptone soy broth, at 30°C. The negative control was not inoculated. Both controls were treated in the same way as the experimental MPN tubes with regard to incubation time and temperature of incubation. After primary incubation at 44.5°C the tubes were examined for the production of gas and media colour change, indicating acid production.

As six dilutions were used in this decimal series of dilutions, the results from only three were used in calculating the MPN. The highest dilution giving a positive result for all five tubes tested and the two next succeeding higher dilutions were used for calculating the MPN index. The MPN index/100 ml was obtained from Table 9221:IV from standard methods (Lasaridi and Stentiford, 1993).

This value was then substituted into the following formula to obtain the MPN faecal coliforms/g sample:

$$\text{MPN faecal coliform/g} = \frac{(10)\ (\text{MPN index/100 ml})}{(\text{largest volume})\ (\%\ \text{dry solids tested})}$$

Salmonella determination was also performed using the multiple tube fermentation techniques. Positives are identified by the presence of gas bubbles in the Durham tubes and the discolouration of the media due to the production of acid. Further confirmation is required for *Salmonella* analysis. This is achieved by transferring positive colonies to triple iron agar slants, incubating the slants at 37°C and re-streaking the positive colonies onto *Salmonella*-specific agar plates. Four series of five tubes were used for the analysis. The first series of five flasks were inoculated with 10.0 g of well-mixed sample per tube (each flask contained 10.0 g of original sample). The second series of tubes received 1.0 g of well-mixed sample (each tube contained 1.0 g of original sample). Using a sterile pipette, 50.0 g of sample were transferred to a sterile screw-cap bottle containing 450 ml of sterile buffered dilution water and mixed by vigorously shaking the bottle a minimum of 25 times. Tenfold serial dilutions were then performed.

The four series of tubes were incubated at 37°C for 48 h. For MPN analysis of *Salmonella*, 10 ml of a wild-type *Salmonella* culture were inoculated as the positive control. This culture was grown overnight at 37°C in tryptone soy broth. After primary incubation, tubes showing acid and gas were presumptive for *Salmonella*. Gas production was indicated by the presence of gas in the Durham tubes, with a colour change in the media indicating acid production. The numbers of positive and negative tubes were recorded.

Presumptive *Salmonella* colonies were streaked on triple sugar iron agar slants for typical appearance, purification

and identity tests. Growth on triple sugar iron agar slants indicates the ability of an organism to ferment lactose, sucrose and glucose with formation of acid and gas and also the ability to produce hydrogen sulphide. Typically *Salmonella* sp. slant cultures, incubated overnight at 37°C gave one of the results shown in Table 4.5.

Positive cultures were purified by transferring them back to brilliant green agar and *Salmonella–Shigella* agar plates for the development of isolated colonies. After streaking, these plates were inverted and incubated at 37°C for a period not exceeding 24 h. Positive incubated brilliant green agar culture plates typically contained clear *Salmonella* colonies surrounded by a brilliant red zone while positive *Salmonella–Shigella* culture plates contained typically clear, pink-edged, black-centred *Salmonella* colonies.

As four dilutions were used in this decimal series of dilutions, the results from only three were used in calculating the MPN. The calculations were based on the same principle as for faecal coliforms which has been previously described. The MPN index/100 ml was obtained from Table 9221.IV (APHA, 1992). The MPN/4 g was calculated according to the following formula:

$$\textit{Salmonella}\ \text{MPN/4 g} = \frac{\text{(MPN index/100 ml)}}{\text{\% dry solids}}$$

4.3.14 Composting sampling protocols

The activator used in the experiment was J.A. Bower's Garotta biological compost maker. This blend of naturally occurring high-activity microbes and enzymes was obtained from a garden centre locally. The study involved the use of 500-l units constructed from recycled plastic.

These aerobic composters are equipped with screw-top covers to protect against rain and perforations were present along the sides of the bins to facilitate airflow. Each composter was balanced on a central axle to facilitate turning. The sitting area of each composter was approximately 1 metre square. Two treatments were tested with one replicate each, giving a total of two composters. The first composter contained a compost activator, with the second bin acting as a control.

The composting material used consisted of approximately 235 l of waste from greengrocers and grass clippings which were added in layers to each composter on Day 0. Both units received the same amounts of each constituent (Table 4.6). To improve structure and C/N ratios, sawdust and cardboard were applied in thin layers between green wastes.

During the course of the composting process, about 50 l of lawn cuttings and 100 l of food waste were added, resulting in a total addition of 400 l organic matter per composter. These additions were made twice during the trial on Day 0 and on Day 30.

Moisture was monitored weekly and adjusted as needed throughout the composting process. Pile temperature was monitored manually using analogue thermometers. Temperature readings were taken daily at the surface, towards the central part of the top, the middle and bottom locations of the piles. For the surface temperatures, the thermometer was plunged into the compost at a depth of 10 cm from the surface of the pile. The compost samples were characterised weekly for the following parameters: water content, C and organic matter contents, foreign matter content, pathogen levels and total Kjeldahl N

Table 4.6. Compost starting constituents.

Sample	Volume (l)	Weight (kg)
Tomatoes	6.0	5.35
Brussels sprouts	10	5.5
Broccoli	7.0	3.05
Grapes	5.0	3.60
Bananas	19	14.3
Sawdust	60	15.2
Bread	44	14.8
Cardboard	84	12.8

Table 4.5. Typical reactions for *Salmonella* identification on triple sugar iron agar slants.

Reaction	Cause
Yellow butt and red slant	Yellow due to fermentation of glucose Red due to limited glucose in the medium
Yellow butt and slant	Fermentation of lactose and/or sucrose, therefore excess acid formation in the entire medium
Red butt and slant	None of the sugars fermented and neither CO_2 nor H_2S produced
Gas formation (H_2S)	Noted by splitting of the agar. Seen by a blackening of the agar

content. The oxygen (O_2) uptake rates were determined at various different time points throughout the trial.

Actively composting material was sampled weekly immediately after mixing on Days 0, 7, 14, 21 and 27 and prior to adding new material on Day 30 (Table 4.7). Composite samples were taken from four locations within each treatment, top section (approximately 100 cm from the base of the pile), central section (approximately 55 cm from the base of the pile) and edges of the composters. Each composite sample represented approximately 1 l of compost. These four subsamples were mixed together to allow one composite sample per compost unit to be analysed.

4.4 Results and Discussion

The key parameter for any composting process is the C/N ratio. According to the FCQAO, C determination should be performed by the method referred to as 'loss on ignition'. The suggested method of the FCQAO is a total Kjeldahl N determination. This was the first test developed and optimised during the validation phase of the project. According to FCQAO standard procedures, the analysis can be performed on either moist or dry samples.

Total Kjeldahl N analysis was carried out on both fresh fruit samples and on dry samples. It was believed that the amount of moisture, protein, carbohydrate, fat and ash content present in the samples when added together would sum to 100%. The ash content of the fruit and vegetables was ascertained using the loss-on-ignition method. Since fruit contains negligible amounts of fat, for the benefit of this experiment, the fat content was assumed to be approximately between 1 and 2%. This allowed for the assumption that the remainder of the material was equal to protein and carbohydrate content. In order to calculate the protein content, the Kjeldahl procedure was carried out on the fruit samples. The samples were examined fresh (i.e. moist) and after they had been dried out at 105°C for 24 h. The results of these analyses are shown in Table 4.8.

It was clear from these results that the use of fresh samples resulted in inaccurate readings being obtained for N content. For this reason, the remaining total Kjeldahl N procedures were carried out using only pre-dried samples, which involved heating at 105°C for 24 h prior to analysis.

To determine if the process was now suitable for use on compost samples, total Kjeldahl N analysis was performed on the two compost samples, 1 and 2, with results presented in Table 4.9.

Next a protocol was developed to use a respirometry apparatus, to determine the respiration rates in compost samples. Since there is no standard method involving respirometry for determining the O_2 uptake rate listed by the FCQAO, a method previously described by Lasaridi and Stentiford (1998) was employed.

Following a number of tests, a sample volume of 70 ml was used for subsequent analysis as this proved to be the maximum amount of compost that could be used that allowed sufficient aeration of the sample.

Table 4.7. Sampling protocol for material during composting.

Sampling days	0, 7, 14, 21, 27, 30
Individual sample unit size	1 l
Number of sampling locations	4
Collection depth from material surface	15–60 cm
Number of individual samples per location	5
Sample collection timing relative to turning	Immediate
Volume of collective sample per treatment	4 l

Table 4.8. Moisture, C and N content of fruit samples.

Sample	Water content (%)	C content (%)	N content moist sample (%)	N content dry sample (%)
Tomatoes	93.5	37.8	36.89	2.52
Grapes	34.8	40.6	63.65	0.47
Bananas	80.6	42.7	26.87	0.98

Table 4.9. Moisture, C and N content of two compost samples.

	N content (%)	C content (%)	Moisture content (%)
Sample 1	1.09	41.57	82.63
Sample 2	2.13	25.16	72.0

Using the newly validated procedure, respirometry was carried out on the two test compost samples (Fig. 4.1) to determine the respiration rate. The O_2 uptake rate for Sample 1 was 75 mg O_2/kg VS/h. The rate for Sample 2 was 82 mg O_2/kg VS/h. Both rates were below the limit of 150 mg O_2/kg VS/h required by current legislation.

In order to evaluate the phytotoxicity of compost samples, cress seeds were grown in varying volumes of compost relative to experiment topsoil.

The topsoil was examined initially to assess its suitability for inclusion in the phytotoxicity test. Three tests were carried out on the soil sample. These were, firstly, an assessment of the soil to determine if it could sustain the growth of the cress seeds essential to the phytotoxicity test; secondly, the soil was then examined for the presence of any seeds that would disturb or inhibit the growth of the cress seeds; and, thirdly, different amounts of the soil were used as potting medium to assess if varying the amount of soil had any effect on the growth of the contained seeds.

To determine if the topsoil could sustain the growth of cress seeds, a known quantity of cress seeds was planted in pots all containing the same amount of topsoil. These pots were then placed in a growth chamber for a period of 7 days and kept at 18°C, while being exposed to fluorescent light for 16 h daily. The moisture content of the topsoil was maintained at 70% to promote the growth of cress seeds. The pots were removed from the growth chamber on Day 7. After removal from the growth chamber, the number of plants that had grown were counted and recorded. Representative results from some of these test pots are shown in Table 4.10.

Table 4.10. Number of plants that grew in the vegetative test for the topsoil.

Treatment	Number of seeds planted	Number of plants present
1	30	29
2	30	28
3	30	30

While carrying out the vegetative test on the topsoil, pots containing topsoil were also placed in the growth room for the same period of time and under the same growth conditions. These pots contained no cress seeds and were used to determine if any seeds were present in the topsoil that could hamper the growth of the cress seeds in a trial situation. After 7 days, the pots were removed from the growth chamber. No growth was recorded in any of the test pots, indicating that no seeds were present in the

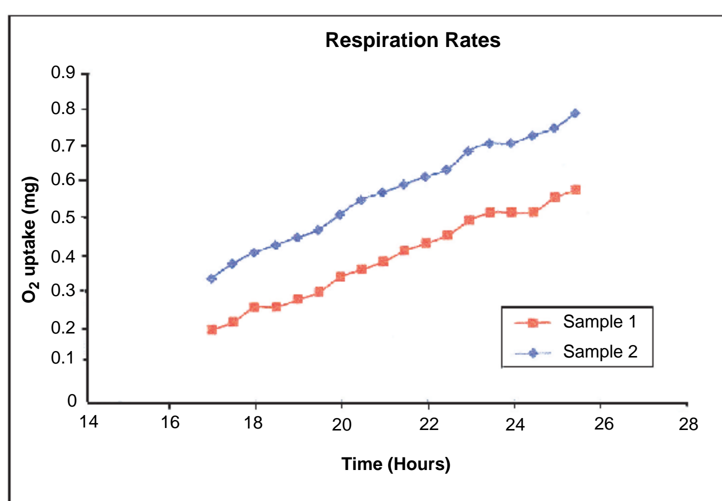


Figure 4.1. Respiration rates for compost Samples 1 and 2.

soil which were capable of germinating during the trial time (7 days) and under the same growth conditions as the trial (18°C, 16 h photoperiod).

To ensure that results obtained from the phytotoxicity experiment were valid and reflected the toxicity of the compost rather than the varying amounts of soil used influencing results, cress seeds were planted in pots each containing varying amounts of topsoil. This would facilitate the determination of the possible effects that the use of varying amounts of soil could have on the resultant growth of plants. The various amounts of soil used in this evaluation experiment are listed in Table 4.11. The same amount of seeds was planted in each pot and the pots were incubated at 18°C for 7 days. After 7 days of incubation in the growth chamber, the number of plants that grew in each pot was counted and then removed from the test pots, weighed and their fresh weights recorded. They were then dried at 105°C in a drying chamber for 24 h. Their dry weights were obtained and compared to investigate the effects, if any, of using varying amounts of soil as a growth medium. While counting the number of plants that germinate in each pot provides valuable information, it does not provide information about the state of the plants. The same numbers of seeds may germinate in all pots but the plants grown in the lesser amounts of soil may not be as robust as plants grown in higher amounts of soil. Therefore in this case it is important to compare the dry weights.

From the results obtained, it was evident that the amount of soil used as the growth medium in the experimental pots had no detrimental effect on the growth of cress seeds. There was no significant difference found between the number of seeds that germinated in each pot and the dry weights of the resultant plants showed that the varying amounts of soil used did not have an adverse effect on their growth.

Following this a cress test was conducted on Samples 1 and 2 to assess their phytotoxicity (Table 4.12). The results obtained clearly indicate that neither compost sample displayed phytotoxic effects on cress seed germination.

Table 4.12. Cress test results for two compost samples.

Ratio	% growth	
	Sample 1	Sample 2
100% soil	100	100
50:50	100	100
60:40	100	100
70:30	100	100
80:20	100	100
90:10	100	100

The next focus was on the analysis of foreign matter, which should not be >2 mm. To ensure that the sieving equipment and shaker were set at accurate settings to facilitate recovery of particles >2 mm, a validation test was performed.

A known quantity of the two dried compost samples (approximately 150 g) was spiked with a known amount of contaminants measuring over 2 mm in dimension (approximately 30 g). The spiked samples were then sieved through the appropriate sized sieve (2 mm) for a known length of time (10 min) and the foreign matter content assessed. To act as a control for the experiment, 150 g of the two compost samples were also sieved through the same sieves for the same time periods. The results of these analyses are given in Table 4.13. Both samples were assessed initially at three different time periods (5, 10 and 15 min) to determine which time period was optimal for foreign matter recovery. The samples were sieved at two different shaker speeds to determine which speed allowed for the greatest recovery of foreign matter. As the results show, different levels of recovery were recorded for the three time periods. Sieving the material for 5 min resulted in reduced levels of recovery compared with 10 and 15 min of sieving. The results for the 10- and 15-min sieving experiments produced the

Table 4.11. Amount of soil used in the determination and evaluation of the content of seeds present and capable of germinating in potting soil.

Treatment	% potting soil in test pots	Number of seeds planted	Number of seeds that grew	Average dry weights of plants (g)
1	100	30	29	1.52
4	70	30	29	1.59
6	50	30	28	1.45

Table 4.13. Percentage particle size recovery for Samples 1 and 2 at varying time points.

	Starting weight (g)	Particles added >2 mm (g)	% particles recovered >2 mm (g)					
			5 min		10 min		15 min	
			Slow	Fast	Slow	Fast	Slow	Fast
Sample 1	181.23	30.14	26.58	10.13	32.79	15.15	32.80	15.20
Sample 2	180.65	30.08	16.79	13.46	31.65	20.45	31.67	20.45

same results. As a result of these findings, the sieving time period of 10 min was used in all subsequent foreign matter determinations.

Efforts were then focused on pathogen determination. In order to successfully recover pathogenic micro-organisms from compost samples, certain criteria need to be initially addressed. To ensure that an isolation method is working correctly one must be certain that the test selects for the desired pathogen and also achieves full and accurate recovery. In order to validate the pathogen recovery methods, the two compost samples were analysed.

Compost Samples 1 and 2 were treated the same way in all experiments carried out throughout the validation process. The compost samples were divided into three sections prior to evaluation. Each subsample of compost was treated differently. The treatments involved are listed in Table 4.14. These treatments were employed in order to provide controls for the experiments.

Table 4.14. Treatments used for the purpose of validation for the enumeration of bacteria.

Subsample number	Treatment used
Subsample 1	Autoclaved sample
Subsample 2	Spiked sample
Subsample 3	Original sample

The autoclaved sample was sterilised by autoclaving the sample at 121°C for 15 min. This was carried out in order to sterilise the compost sample, thereby removing all bacteria in the samples. The second subsample was inoculated with the relevant bacterial cultures. By inoculating the samples with the relevant cultures the amount of bacterial cells present are increased considerably. Incubation of these ‘spiked’ samples, should result in approximate 100% recovery of bacteria. This spiked sample acted as a second control for the validation procedure. The third subsample examined as part of the validation process was the original sample. This sample was not treated in any way. This sample, when inoculated, will give an accurate determination of pathogens present in the samples. To achieve this, 1 kg of the compost sample was divided into two equal samples. One sample was autoclaved and the second sample was further divided into two equal subsamples. These two subsamples made up the final two parameters examined in the validation procedure. The first subsample was spiked with the relevant bacterial culture and the second subsample was not treated in any way. The three samples (autoclaved, spiked and original) were used as inoculation for two MPN experiments each. Of the two experiments set up for each treatment, one set was incubated at 37°C while the second set was incubated at 44.5°C. Incubation of MPN tubes at temperatures of 37°C allowed for the isolation of total coliform organisms, while incubation at 44.5°C selected for only faecal coliforms.

4.4.1 Bacterial pathogen analysis

Table 4.15 outlines the pathogen levels observed as assessed using Rappaport–Vassiliadis, which is a selective enrichment broth used for the identification of *Salmonella*, designed to exploit four characteristics of *Salmonella* species: the ability to survive at relatively high osmotic pressures, to multiple at relatively low pH, increased resistance to malachite green and relatively less demanding nutritional requirements. The autoclaved, spiked and original samples were incubated at the stated temperature for 48 h. The results reflect six MPN analyses.

For the autoclaved treatment, no *Salmonella* cells were recovered. The spiked samples incubated at 44.5°C showed no recovery of *Salmonella* sp. These results were due to the fact that the incubation temperature of 44.5°C was too high to allow for growth and therefore subsequent recovery of *Salmonella* sp. Full recovery of *Salmonella* sp. was obtained for the spiked Samples 1 and 2 incubated at 37°C. All the MPN tubes used in the spiked treatment incubated at 37°C gave positive results, thereby attaining

Table 4.15. Pathogen levels for the autoclaved, control and spiked compost samples using Rappaport–Vassiliadis broth media.

	Autoclaved sample		Spiked sample		Original sample	
	37°C	44.5°C	37°C	44.5°C	37°C	44.5°C
Sample 1	0	0	>581	0	12	0
Sample 2	0	0	>581	0	327	0

the highest MPN/4 g value possible, i.e. >581 MPN/4 g of total solids. Results obtained for the original samples incubated at 44.5°C were zero for both compost Samples 1 and 2. This was due to the inability of *Salmonella* to grow at 44.5°C. The result for Sample 1 of the original treatment showed that 12 MPN/4 g of total solids was present in the sample. Values of 327 MPN/4 g of total solids were recorded for Sample 2 in the original treatment incubated at 37°C. These *Salmonella* recovery results were higher than the maximum limits allowed for *Salmonella* content recommended by the Irish EPA (<3 MPN/4 g of total solids). This would indicate that either the temperature in both composting processes failed to reach the high temperatures (70°C) required for sterilisation or that the high temperatures were attained but not maintained for the required period of time (e.g. 15 days for windrow composting) to reduce pathogen numbers. Thus the MPN method had been validated for the isolation of *Salmonella* species.

Next, efforts focused on the validation of the MPN method used for recovery of faecal coliforms, which involved examining the selective media used and the temperatures employed in the isolation process. The selective media used for this part of the validation experiment was A-1 medium, which is routinely used to isolate faecal coliforms. This broth was sterilised and dispensed into the appropriate MPN tubes.

Results obtained are shown in Table 4.16. As might be expected autoclaved samples at both incubation temperatures were negative. Recovery of coliform organisms was achieved for both spiked samples at both incubation temperatures. The results obtained for spiked Samples 1 and 2, incubated at 37°C (total coliforms), were higher than those obtained for the same samples incubated at 44.5°C (faecal coliforms). This result was mirrored for the original samples. For Sample 1, total coliform numbers were calculated as 2.1×10^6 of which 1.4×10^4 were faecal coliforms. For Sample 2, 1.6×10^5 MPN/g of total solids were deemed faecal coliforms of the total number of 3×10^6 MPN/g of total solids isolated. These high levels of faecal coliforms present in both samples further highlight the theory that the composting process employed to produce these samples did not either reach or maintain temperatures required to reduce the levels of pathogens present to below the recommended levels, which are <1,000 MPN/g of total solid for faecal coliforms and <3 MPN/4 g of total solids for *Salmonella* sp.

4.4.2 Pilot-scale composting unit

The second task in this project was to design and construct a pilot-scale composting unit. It was decided to use 500-l household composters, constructed of recycled plastic, with a sitting area of approximately 1 metre square. These composters are marketed as tumbler-type composters and are equipped with screw-top covers to protect against rain, with perforations present along the sides to facilitate airflow. Two treatments were tested. The first composter contained a compost activator, while the second acted as a control. Approximately 235 l of waste from greengrocers and grass clippings were added in layers to each composter. Both compost units received the same amount of each constituent (Table 4.17). To improve structure and C/N ratios, sawdust and cardboard were applied in thin layers between green wastes. The objectives here were twofold – firstly to examine the effects of using certain blends of organic materials as

Table 4.16. Pathogen levels for the autoclaved, control and spiked compost samples.

	Autoclaved sample		Spiked sample		Original sample	
	37°C	44.5 °C	37 °C	44.5 °C	37 °C	44.5 °C
Sample 1	0	0	4.5×10^6	3.6×10^6	2.1×10^6	1.4×10^4
Sample 2	0	0	5×10^6	3.7×10^6	3×10^6	1.6×10^5

Table 4.17. Compost constituents.

Sample	Volume (l)	Weight (kg)
Tomatoes	6.0	5.35
Brussels sprouts	10	5.50
Broccoli	7.0	3.05
Grapes	5.0	3.60
Bananas	19	14.3
Sawdust	60	15.2
Bread	44	14.8
Cardboard	84	12.8

feedstocks and secondly to determine the effects of adding an activator to the process. During the course of the composting process, about 50 l of lawn cuttings and 100 l of food waste were added resulting in a total addition of 400 l organic matter per composter. Actively composting material was sampled weekly and adjusted as needed throughout the composting process. Samples were taken weekly after mixing on Days 0, 7, 14, 21 and 27 and prior to adding new material on Day 30 (Table 4.18). Composite samples were taken from four locations within each treatment, top section (approximately 100 cm from the base of the pile), central section (approximately 55 cm from the base of the pile) and edges of the composters. Each composite sample represented approximately 1 l of compost. These four subsamples were mixed well together so that one composite sample per composter was available.

Throughout this trial, three separate parameters were monitored, namely temperature, moisture and respiration rate. Temperatures reached during composting are an invaluable indication of the effectiveness of the compost process. Temperatures in both compost units fluctuated throughout the trial, indicating that varying degrees of microbial decomposition were occurring within the compost (Fig. 4.2), with the temperature being slightly higher overall in the control unit. Moisture levels were also assessed, where an initial drop was observed up until Day 15, with levels increasing up to 60–70% thereafter (Fig. 4.3).

Finally, respiration rates were also assessed throughout this period as an indication of overall microbial metabolic activity. Much higher levels were observed after 21 days as opposed to 14 days, while respiration rates were higher in each case in the control samples (Fig. 4.4).

Carbon and nitrogen levels in the compost were also assessed to allow insights to be gained into the rate of decomposition being achieved and thus how ‘finished’ a compost may be with respect to raw or mature composts. Again, both compost units were analysed, one which contained a compost activator, and the second which acted as a control (Fig. 4.5a and b).

The recommended C to N ratio for composting is approximately 30:1. In order to obtain this starting C to N ratio, sawdust was added not only to provide C but also to act as a bulking agent to facilitate airflow within the compost pile and to maintain favourable aerobic conditions. A number of problems arose however due to the addition of this C source. Sawdust, which is mainly produced from old trees, is rich in lignin, which is not easily degraded and therefore may not be suitable for the short-term composting process. In addition it is extremely dry, therefore when in contact with a moisture source it will markedly affect the available moisture.

The reduction in C content of the control bin, while detectable, was not significant. The C content was recorded at 43% on Day 8; this remained virtually constant throughout the remainder of the trial, being around 41% between Days 8 and Day 30. The C content for the activator compost unit was quite similar, being 41% on Day 7, before dropping to 36% by Day 30. The N levels of both bins followed similar patterns, with levels in the control compost unit remaining at 3.5% between Days 8 and 30. The N content of the activator compost unit was at 7% at the beginning of the trial but this was reduced to less than 1% by Day 30. Both compost units were

Table 4.18. Sampling protocol for material during composting.

Sampling days	0, 7, 14, 21, 27 and 30.
Individual sample unit size	1 l
Number of sampling locations	4
Collection depth from material surface	15–60 cm
Number of individual samples per location	
Volume of collective sample per treatment	4 l

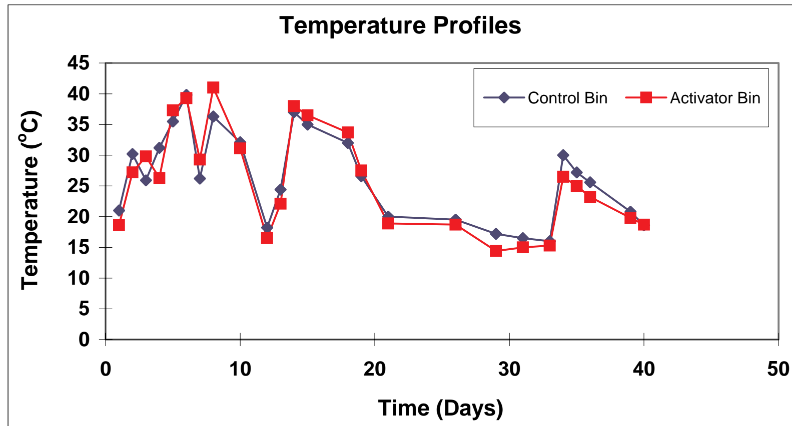


Figure 4.2. Temperature profiles for control and activator compost units.

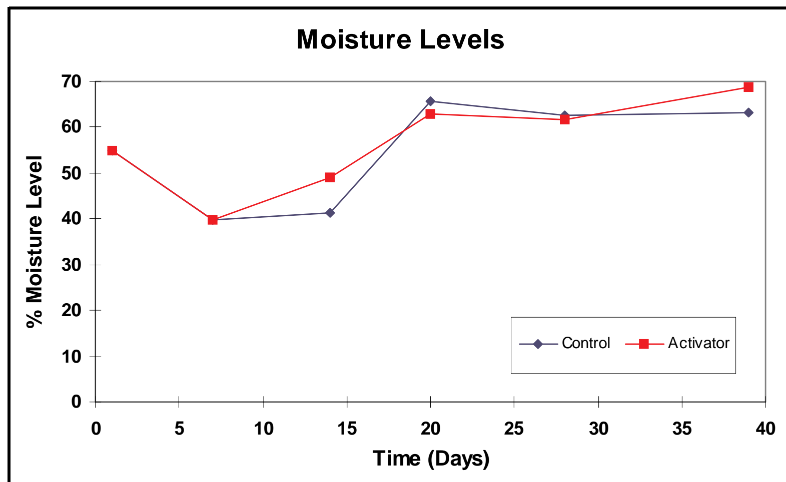


Figure 4.3. Moisture profiles for control and activator compost units.

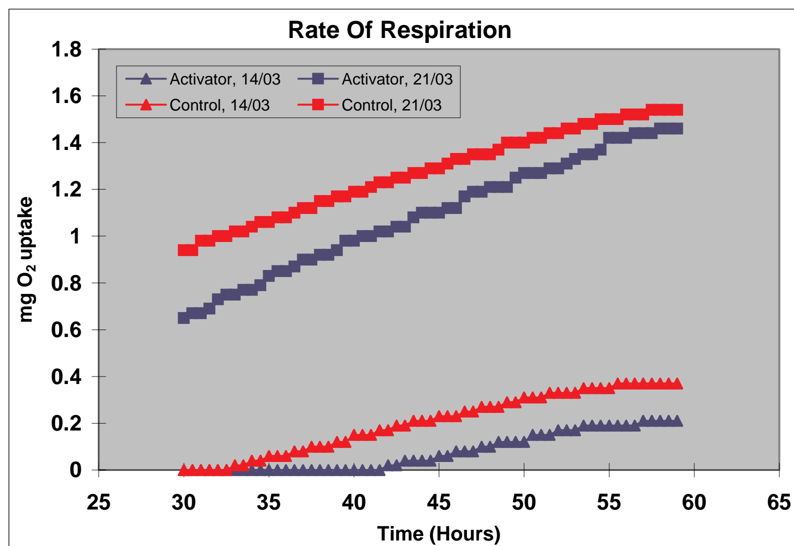


Figure 4.4. Respiration rates for control and activator compost units.

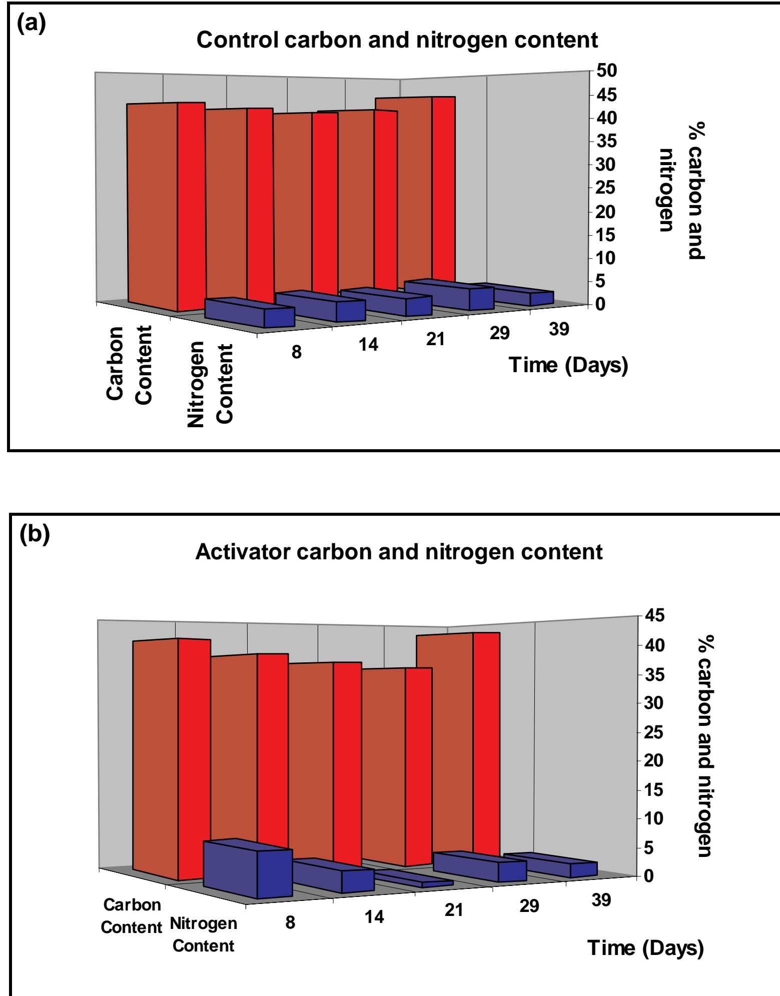


Figure 4.5. (a) Carbon and nitrogen content profile for control compost bin. (b) Carbon and nitrogen content profile for activator compost bin.

reseeded on Day 30 and the effect of the introduction of fresh feedstock is evident by an increase in C and N in both units on Day 30. The majority of the fresh waste (freshly cut grass, fruit and vegetable waste) was high in N content and this is evident from both graphs (Fig. 4.5a and b). Carbon levels rose slightly after Day 30. After the initial increase no further fluctuations in C content were observed.

It appears likely that these results indicate a lack of available N and C. Since neither the N nor C levels changed between Days 21 and 25; it can be concluded that the detectable N and C is largely unavailable to the microbial populations within the compost pile. It also appears likely that during the first 30 days all the readily available nutrients may have been utilised, thus leaving limited nutrients available for the predominant microbial decomposing populations present in the pile after a certain period of time.

The particle size was also measured throughout this study, being recorded once a week,throughout the trial, to estimate the reduction in size of the raw material as it was being decomposed (Fig. 4.6), with material measuring >2 mm in diameter being expressed as a percentage of the total volume of material examined. The particle size results for both the control and the activator compost units mirrored the C and N findings, with no further decrease in particle size being observed following an initial reduction in values, suggesting that the decomposition process had in some way been hampered, since reductions in particle sizes are often a good indication as to the speed at which the decomposition process is proceeding within a compost unit (Chang *et al*., 2006).

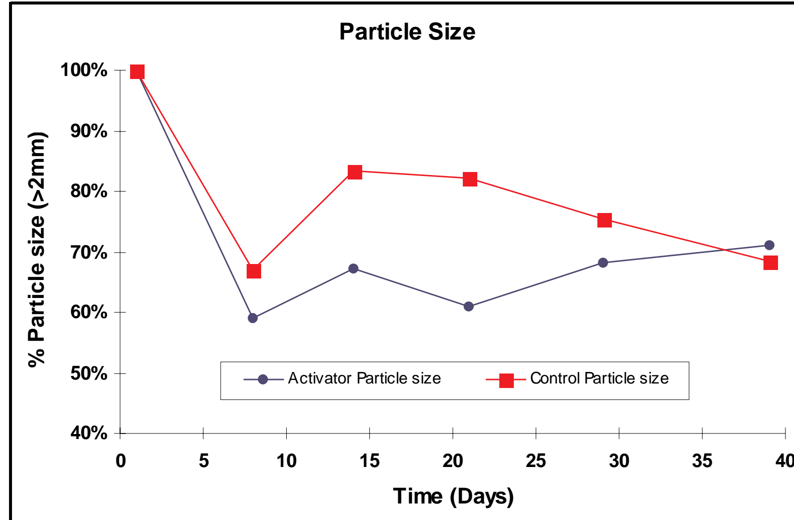


Figure 4.6. Percentage particle size for the control and activator compost bins.

4.5 Conclusions

Composting is an accelerated bio-oxidation of organic matter passing through a thermophilic stage (46–65°C) where micro-organisms (mainly bacteria, fungi and actinomycetes) liberate heat, CO_2 and water. The focus of this project was to evaluate various materials and methods to test and validate all four areas used in the evaluation of compost quality. The various methods employed included ICP-MS, total Kjeldahl N determination and MPN analysis. The overall aim was to standardise the various quality testing methods, and to then apply these protocols to samples from different sources. The results obtained from the commercial compost samples highlighted the need for validation of testing methods to ensure their suitability and allowed for the optimisation and adaptation of the testing procedures for use with compost samples, until a complete set of tests was developed that were suitable for analysing the quality of compost samples. Analysis of the laboratory-scale test compost units showed the benefits of monitoring the process using the validated testing methods.

5 Project 4: Investigate the Key Operational Parameters Involved in the Biological Nutrient Removal (EBPR) Process, while Treating a Food Processing Waste-Water Stream

5.1 Project Overview

One of the key environmental problems facing the Irish food industry is the treatment of large-volume waste-water streams, containing N and P. These nutrients are considered to be the main precursors of surface-water eutrophication, having the potential to promote excessive growth of algae (Danalewich *et al*., 1998). Phosphorus is the key environmental factor in effluents from the Irish food industry, particularly in the dairy sector. Traditionally, PO_4 removal from waste-water streams has been carried out by chemical precipitation (Stratful *et al*., 1999). Such techniques, although reliable, are both expensive (due to the high cost of chemicals and the need to handle, treat and dispose of additional quantities of chemical sludge) and may also have a detrimental effect on effluent quality (Lee *et al*., 1996). An alternative to chemical precipitation is that of BPR which exploits the potential for micro-organisms to accumulate PO_4 (as intracellular polyphosphate) in excess of their normal metabolic requirements (Brdjanovic *et al*., 1998; Mino *et al*., 1998). The BPR process is primarily characterised by circulation of activated sludge through anaerobic and aerobic phases, coupled with the introduction of influent waste water into the anaerobic phase (Mino *et al*., 1998). In the anaerobic phase, sufficient readily degradable C sources, such as VFAs, must be available to induce the PO_4-removing bacteria to take up the acids and release PO_4 into solution (Morse *et al*., 1998). In the aerobic phase, luxury P uptake occurs, which results in overall P removal to as much as 80–90% (Morse *et al*., 1998). Incorporation of an anoxic phase permits the combined removal of N and PO_4 from the process waste waters (Metcalf and Eddy, 1991; Crites and Tchobanoglous, 1998).

A pilot-scale study was conducted at the Kerry Ingredients Plant, Listowel, Co. Kerry, Ireland, to investigate the key operational parameters involved in the BNR (combined N and P removal) process, while treating a waste water emanating from the food ingredients factory. Kerry Ingredients, Listowel, is a dairy-processing company that produce casein and whey products. It produces flavoured milks, milk protein isolates, dairy spreads, cheese, and a variety of flavourings and other food ingredients. The waste waters emanating from this process contain various forms of carbohydrates, proteins and lipids. A preliminary pilot study was performed for 4 months in 1997, from July to October. Following significant increases in the production levels at the plant, resulting in higher concentrations of nutrients being present in the waste waters treated at the on-site waste-water treatment plant, it was decided to conduct a second pilot trial in 2001–2002 with a pilot-scale reactor of similar design; however, some cost-saving alterations were incorporated to the 2001 design. The two main aims of this study were (i) to determine whether the 2001 BNR system could perform satisfactorily under the higher nutrient loading conditions, and (ii) to compare the relative performance of the 1997 and 2001 design, with the 2001 system designed to operate under a more cost-effective regime.

5.2 Experimental Set-Up

The pilot trials took place on site in the Kerry Ingredients Plant, in Listowel, Co. Kerry. The pilot reactors were operated in an outdoor location, without any temperature control. Therefore operating conditions reflected the seasonal temperature variation that occurs in the south-west of Ireland. The average daily temperature for the duration of the trial was 18°C, with the highest and lowest temperatures being 23°C and 10°C, respectively. Both the 1997 and the 2001 pilot plants had a similar configuration as shown in Fig. 5.1. However, they differed in the sizing of each zone and the recycle rates. In both systems, each zone was separated by movable baffles to allow the volume of each to be varied. The waste water flowed by gravity from the anaerobic to the anoxic and to the oxic zones. Post-aeration, the mixed liquor flowed into a 1,040-l clarifier, which was equipped with a slow mixing scraper and a baffle driven by a 1-rpm motor. Each zone

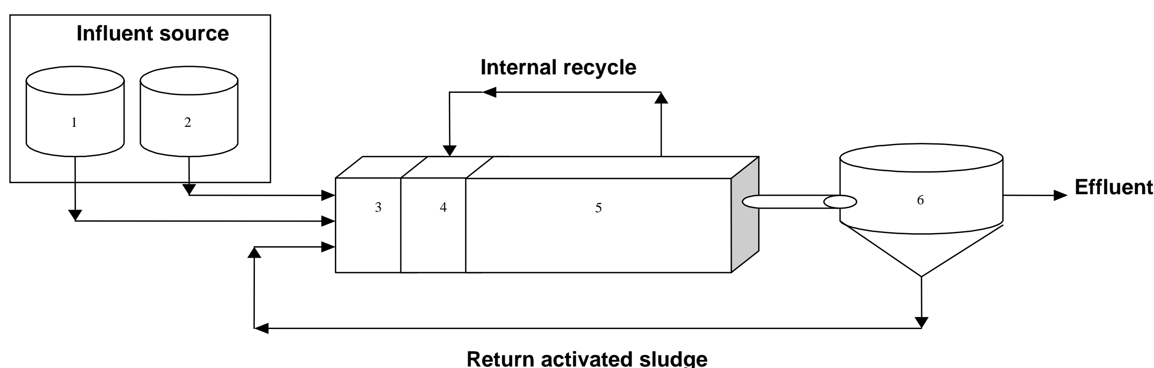


Figure 5.1. Schematic of the A/A/O BNR pilot plant. Key: 1, interstage basin; 2, primary acidogenic fermenter; 3, anaerobic zone; 4, anoxic zone; 5, oxic zone; 6, clarifier.

contained a submersible Lotus Diamond pump, which provided a continuous mixing of the reactor contents. Air was supplied to the oxic zone using a Rietschle SKG 200-2 air blower. The pumping of feed and recycled material was performed using Watson Marlow 604S and 405S peristaltic pumps. The 1997 anaerobic/anoxic/oxic (A/A/O)-type pilot reactor was a 936-l stainless steel rectangular tank containing anaerobic (156 l), anoxic (156 l) and oxic (624 l) zones. The 2001 A/A/O-type pilot reactor was a 600-l stainless steel rectangular tank containing anaerobic (60 l), anoxic (60 l) and oxic (480 l) zones. The influent flow rate (*Q*) to the 1997 and 2001 pilot plants was maintained at 720 and 504 l/day, respectively. The return activated sludge (RAS) flow rate from the clarifier to the inlet of the anaerobic zone was maintained at 2*Q* (1,440 l/day) and 0.9*Q* (454 l/day), for the 1997 and 2001 systems, respectively. The internal recycling (IR) from the end of the aerobic zone to the inlet of the anoxic zone was at a value of 4*Q* (2,880 l/day) for the 1997 trial and 1*Q* (504 l/day) in the 2001 trial. The anaerobic HRT for the 1997 and 2001 systems was maintained at 5.2 and 2.9 h, respectively, resulting in the total HRT for the anaerobic, anoxic and oxic zones in both systems of 31.2 and 28.6 h, respectively. The solids retention time (SRT) was maintained at between 10 and 15 days for both systems.

5.2.1 Primary acidogenic fermenter (PAF)

The same side-stream fermenter was employed to generate elevated levels of VFAs for both studies. The PAF was a 1,040-l enclosed circular tank that contained a mixing baffle driven by a 1-rpm motor to provide gentle mixing of the reactor contents. Sample ports were located on the top and side of the reactor. The PAF was operated in SBR mode, with 3 × 8-h cycles/day. Each cycle consisted of the following sequence: fill (10 min), mixing (360 min), settlement (100 min), decant (10 min), idle (10 min). Note that the first 10 min of mixing coincided with the 10 min of fill time. The content of the PAF while mixing was 948 l, while the reactor volume after decant was 665 l. The feed volume to the PAF was 283 l/cycle, which corresponds to a daily feed volume of 849 l/day and a PAF HRT of 27 h. Solids were randomly wasted from the reactor to maintain an MLSS level in the range 2,000–4,000 mg/l.

5.2.2 Waste-water characterisation

Figure 5.2 outlines the process flow of the full-scale waste-water treatment plant in Kerry. The process flow varies depending on whether it is the high production or low production season. During the high production season, the raw waste water (*Q*) is passed through the dissolved air flotation (DAF) system and collected in a balancing tank; 0.9*Q* from the balancing tank is pumped through the anaerobic digester, with the remaining 0.1*Q* bypassing the anaerobic digester and being treated in the biotower before being collected in the interstage basin. However, in the low production season, the anaerobic digester is decommissioned resulting in a change of the process flow. During the low production season, all of the waste water is treated in the DAF and collected in the balancing tank. However, in the low production season, 0.8*Q* from the balancing tank is sent directly to the interstage basin, with the remaining 0.2*Q* being treated in the biotower before entering the interstage basin. In the pilot-scale plant, the waste water used to feed the PAF came directly from the Kerry Ingredients raw waste-water equalisation basin. A portion of the acidified effluent from

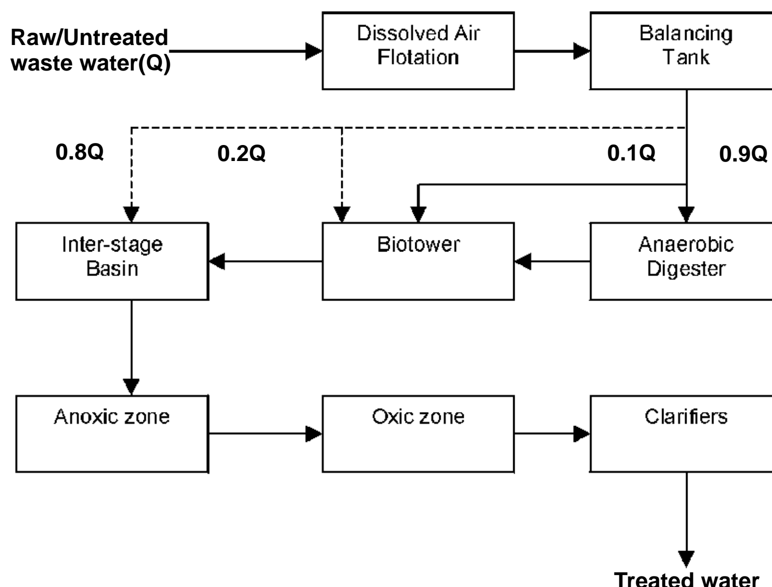


Figure 5.2. Schematic of the Kerry Ingredients full-scale waste-water treatment plant, which is not currently designed to support full-scale biological P removal (the dotted line represents the process flow during the low production season).

the PAF reactor was then fed to the anaerobic zone of the BNR pilot plant. The remainder of the feed to the BNR pilot plant consisted of effluent emanating from the interstage basin on site at Kerry Ingredients (Fig. 5.2). This basin received effluent, which had passed through three stages of treatment (DAF, anaerobic digestion and biotower treatment), in addition to some of the raw waste water, which bypassed the anaerobic digestion treatment stage (Fig. 5.2). The reason for bypassing some of the raw waste away from the digester at full scale was to ensure that some COD would be available for the denitrifying bacteria in the anoxic zone. The ratio of interstage effluent to PAF effluent in the BNR feed to the pilot plant was kept at a constant value of 4:1 in the 1997 trial; however, it was varied during the course of the 2001 trial as will be discussed in the results section. A comparison of the characteristics of the waste-water mixture that was treated by the two BNR pilot plants is shown in Table 5.1.

5.2.3 Seed sludge source

The sludge used to seed the BNR pilot plants on both occasions was obtained from the aerobic basins of the full-scale plant at Kerry Ingredients. The PAF reactor was seeded with sludge taken from the RAS line in the full-scale plant.

5.2.4 Analysis

Samples were taken five times per week for analytical purposes. pH and temperature were monitored using a WTW pH 340 portable pH meter. Analyses for COD, PO_4-P, NH_4-N, and NO_3-N were determined using a HACH DR/2010 spectrophotometer, using reagents and chemicals supplied by the manufacturer. Determination of MLVSS and MLSS was performed in accordance with APHA (1992).

5.3 Results and Discussion

5.3.1 Pilot-plant start-up and operating performance during the 2001 peak season at Kerry Ingredients

At the Kerry Ingredients Plant in Listowel, the peak production period begins at the start of April and ceases at the end of October. Therefore, operation of a waste-water treatment plant under this regime can prove problematic as sustained periods of low organic loading (mid-November–March) are followed by sustained periods of high organic loading (April–mid-November), with additional peaks and troughs of loading occurring within any given week. During peak production season, the anaerobic digester is operational at the full-scale plant to treat the high organic loads that are received during this period. Once the volume of milk intake to the plant decreases during the month of November, the anaerobic

Table 5.1. Comparison of BNR performance during the 1997 and 2001 trials.

Parameter	1997 pilot trial	2001 pilot trial
COD influent (mg/l)	1,008 (2,274; 931)	1,757 (3,210; 555)
COD effluent (mg/l)	43.3 (295; 20)	102.7 (957; 16)
COD % removal	93.3	94.3
NH-N influent (mg/l)	30.1 (71,8; 3,9)	61.9 (107,5; 5.0)
NH-N effluent (mg/l)	0.3 (20,4; 0.1)	2.21 (31; 0.1)
NH-N % removal	98.7	96.7
P influent (mg/l)	36.7 (54; 10)	57 (93,0; 9.3)
P effluent (mg/l)	0.5 (8.5; 0.1)	17 (63.0; 0.39)
P % removal	98.2	74.8
COD/P influent	39.8	36.8
Anaerobic HRT (h)	5	2.85
Total HRT (h)	31.2	28.5
Interstage/PAF influent	4:1	4:1 (1.8:1)
RAS/influent	2:1	0.9:1
Internal recycle/influent	4:1	1:1
F/M (mg COD/mg MLVSS/day)	0.26	0.28
SRT (days)	10–15	10–15

Values in parentheses indicate upper and lower extremes.

digester is no longer required, and is therefore decommissioned until the next peak season in April of the following year.

The 2001 pilot trial began in mid-May, therefore peak production period was under way and the microbial populations in the pilot plant had no acclimation period under lower organic loading in order to acclimate to the new cyclic exposure of anaerobic, anoxic and oxic conditions. The plant operated very efficiently in terms of both COD removal (96%) and soluble N removal (94%) as can be seen in Figs 5.3 and 5.4, respectively. It is evident from Fig. 5.3, that the concentration of the waste water in terms of influent COD was very variable throughout the course of the trial. The highest recorded influent COD was 3,230 mg/l on the 5th August, whereas the lowest value of 350 mg/l was obtained on the 26th November. The

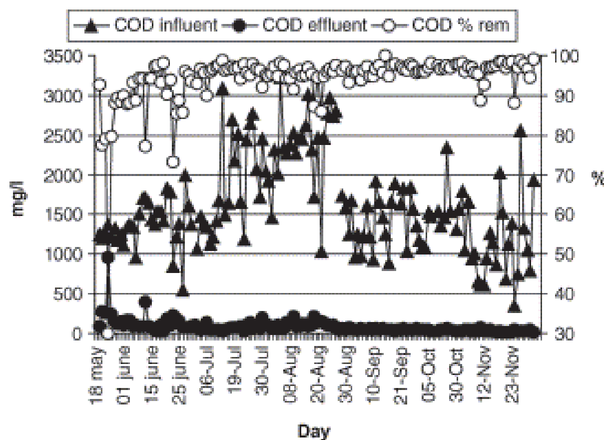


Figure 5.3. The removal of COD by the 2001 BNR system.

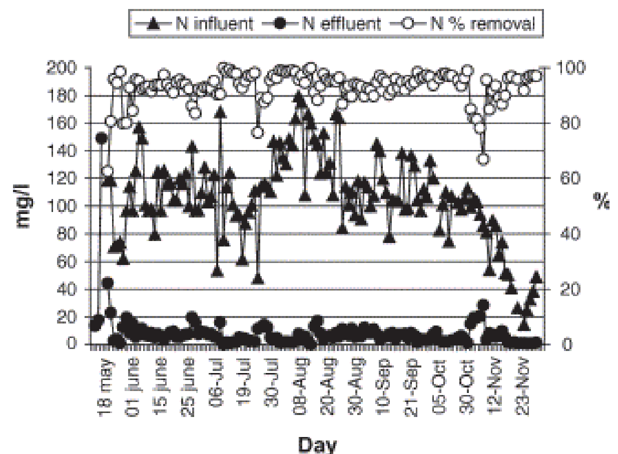


Figure 5.4. The removal of soluble N (NH_3 + NO_3) by the 2001 BNR system.

combined soluble nitrogenous concentration in the waste water consisted of the sum of the NH_4-N and NO_3-N present in the waste water. Throughout the peak production season, while the anaerobic digester was in operation, the nitrogenous loading to the BNR plant was quite high with an average combined soluble NH_3 and NO_3-N value of 107 mg/l entering the system (Fig. 5.4). This was reduced to a mean effluent value of 6.3 mg/l (combined $NH_3 + NO_3$-N) for the duration of the peak period. This very efficient level of removal was obtained over a temperature range of 10–22°C. Once the anaerobic digester was decommissioned in the off-peak season (mid-November), the average soluble nitrogenous concentration in the influent to the plant dropped to 30.6 mg/l with effluent values of 1.4 mg/l being recorded during this period (Fig. 5.4). This was a very satisfying level of performance as nitrification has been reported to be especially sensitive to winter temperatures (Helmer and Kunst, 1998; Rothman, 1998; Wagner *et al*., 1998) and this period coincided with the end of November and the month of December.

The acclimation of P-accumulating bacteria (PAB) became evident after the first 30 days of operation with an anaerobic P release being observed, which was maintained for the remainder of the trial. The characteristic anaerobic P release by the PAB was accompanied by luxury P uptake during the first 2 months of the trial, on occasion reaching >60% P removal (Fig. 5.5). Acclimation took longer than expected, due to

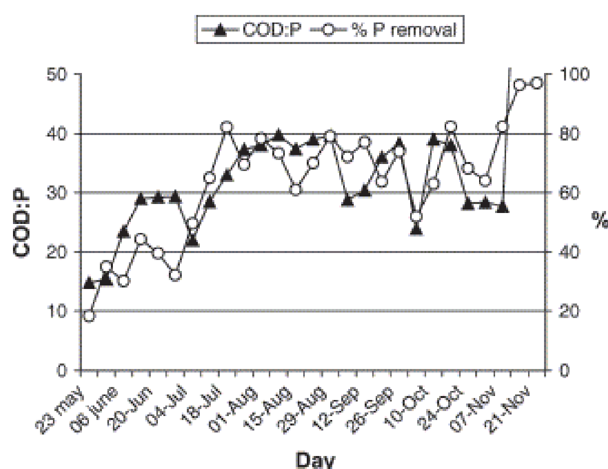


Figure 5.5. A comparison of the relationship between percentage P removal of influent PO_4 with the COD/P ratio of the BNR influent.

problems experienced with the new aeration equipment in the first month of the trial.

However, this upward trend in P removal was difficult to maintain due to insufficient available COD to support both N and P removal. The system was achieving a very high level of nitrification, therefore producing a high concentration of NO_3. Denitrification of the high N load imposed a significant demand on available COD, leading to competition between the denitrifying bacteria and PAB. The COD/P ratio in the influent to the BNR system was approximately 29:1 for the first 2 months of the trial (Fig. 5.5). Randall *et al.* (1992) reported that the minimum COD/P ratio to obtain successful BPR was 40:1; therefore it was clear that the 2001 BNR pilot-plant system contained insufficient COD to remove the high concentration of PO_4 in the influent. Therefore, the BNR feeding regime was modified to increase the influent COD/P ratio. At the start of the trial, 20% of the BNR feed consisted of PAF effluent with 80% consisting of interstage effluent. Interstage effluent had a low COD concentration owing to the fact that it had passed through three stages of treatment, i.e. DAF, anaerobic digestion and through the biotower (Fig. 5.2). Therefore, in July, the percentage of interstage effluent being fed to the pilot plant was reduced from 80% to 64%; the PAF effluent contribution remained at 20% of the total feed, and the remaining 16% consisted of raw waste water taken directly from the raw waste-water equalisation basin, which had not undergone any form of biological treatment (Fig. 5.2). This waste water had a higher COD concentration; however, this new mixture of BNR feed only managed to produce an influent with an average COD/P of 35:1 (Fig. 5.5), as the PO_4 concentration in the waste water at this time also increased (Fig. 5.6). This reflected difficulties that could arise under full-scale operation. Therefore, the suggested ratio of 40:1 was not achieved under this feeding regime until the end of the trial (Fig. 5.5). Hence, the percentage of P removal could not be maintained at a value greater than 80% for any sustained period of time. Figure 5.3 shows that the COD loading to the plant was greatly reduced from the end of August to the end of the trial. However, this reduction was not evident in the soluble N loading to the plant with average influent values greater than 100 mg/l for combined NH_3 and NO_3 (Fig. 5.4) and average influent P values for the same period ranging between 40 and 60 mg/l (Fig. 5.6). The highest influent P concentrations were

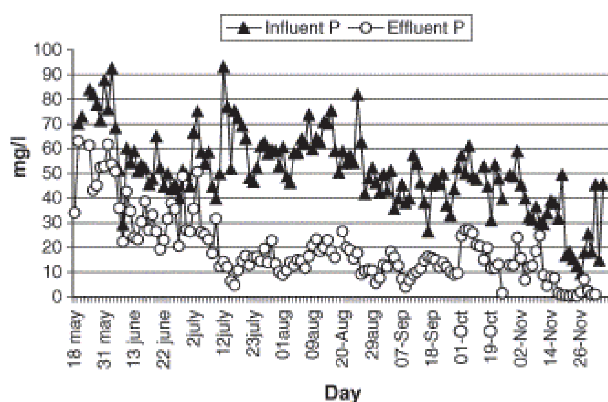


Figure 5.6. The influent and effluent P concentrations recorded in the 2001 BNR pilot trial.

recorded during the months of July and August, with influent values ranging between 60 and 80 mg/l (Fig. 5.6).

Phosphorus removal was retarded on occasion primarily due to mechanical problems. On two occasions, a significant reduction in the MLSS of the system occurred resulting from falling leaves blocking the outlet pipes in the pilot plant. This resulted in the food/micro-organism (F/M) ratio increasing to values as high as 0.5 mg COD/ g MLVSS/day, which is well above the suggested F/M for BNR systems of 0.15–0.25 mg COD/mg MLVSS/day (Metcalf and Eddy, 1991). One of the negative effects of having a very high F/M is that all of the available C may not be sequestered in the anaerobic phase by the PAB, thereby reducing the competitive advantage of these populations over non-PAB. The solids concentration in the system was allowed to increase which gradually reduced the F/M to acceptable values. Reduced P removal was also observed on certain days due to difficulties with the pilot-plant equipment, such as electrical failure with the pumps, and mixers becoming clogged, again due to the presence of fallen leaves in the pilot plant. Such circumstances are not typical of what may arise in a full-scale plant, and are largely a consequence of dealing with a small system with small pipes, small equipment and little buffering capacity. Overall, during the peak season, although the influent PO_4 to the plant was quite high, a satisfactory level of P removal (74%) was obtained, as demonstrated in Fig. 5.6.

5.3.2 *Pilot-plant performance during the off-peak (winter) season at Kerry Ingredients*

In the off-peak season of the trial (from Day 175 onward), the sole C source for the BPR bacteria came from the interstage waste water. Although this waste water was not treated in the PAF prior to being fed to the plant, an average influent P concentration of 24 mg/l was reduced to 1.03 mg/l in the final effluent, which corresponds to a 96% P removal efficiency (Fig. 5.6). One of the concerns at the start of this pilot trial was how the BPR bacteria would perform over the winter period following the decommissioning of the anaerobic digester, with the concomitant reduction in the load to the treatment plant. The data presented show how well these systems responded to this situation, even without PAF effluent being added to the system. One of the reasons for this improved performance in the off-peak season is due to the decommissioning of the digester, which has a high concentration of NH_3 in the effluent. Thus, with less NH_3 entering the pilot plant, less denitrification was required. This ensured that sufficient COD was available for the PAB to remove the PO_4 loads at this time of year. In Fig. 5.5 it is clear that in the off-peak season (mid-November to the end of the trial) the COD/P increased to a value greater than 40:1 for the first time in the trial period. The COD/P values recorded for the last 3 weeks of the trial were all in excess of 60:1, resulting in a percentage P removal efficiency of greater than 95% (Fig. 5.5).

It is clear from this study that the BNR system was capable of treating very high concentrations of COD, soluble N and PO_4, and is easily capable of complying with the Kerry discharge limits for COD, NH_4-N and NO_3-N (150, <1, and <20 mg/l, respectively). However, under the current pilot regime and with the variability in COD loading the system was not capable of complying with the stipulated discharge limit of 1 mg P/l.

5.3.3 *Comparison between the 1997 and 2001 pilot trials*

Table 5.2 illustrates the difference in design between the two A/A/O pilot reactor systems. In Table 5.2 it is clear that the 2001 influent concentrations of COD, NH_4-N and P were approximately twice as high as those entering the 1997 pilot plant. The average effluent values for each of these parameters were also considerably higher than those of the 1997 trial. In terms of COD and NH_4-N removal, both pilot plants were under the permissible discharge limits for the Kerry Ingredients Company. Denitrification was not a problem in either system, with NO_3 values not exceeding 10 mg/l in the final effluent. It appears that the 2001 design was inferior to the 1997 design in terms of P removal. However, to draw a correct

Table 5.2. Comparison of design ratios for the two pilot plants.

Volume	1997 pilot design	2001 pilot design
Total pilot plant volume (l)	963	600
Anaerobic volume (l)	156 (16.66)	60 (10)
Anoxic volume (l)	156 (16.66)	60 (10)
Aerobic volume (l)	624 (66.66)	480 (80)
Primary acidogenic fermenter (m^3)	1.04	1.04

Values in parentheses are percentage of total pilot plant volume.

conclusion on P removal performance, the variation in flow and loading rate to the two plants must be considered, as the 2001 system was operating on a shorter HRT (Table 5.1); therefore this system received a higher loading rate, relative to its size, than the 1997 system. However, in terms of influent COD/P, F/M and SRT values both systems were similar.

To compensate for the differences in flow and loading to both systems an overall mass balance analysis facilitates a more accurate comparison of performance (Table 5.3). The 1997 system received a P loading of 19,224 mg P/day and succeeded in removing 18,864 mg/day from the waste water. Expressing this removal rate per g MLSS in the system equates to a specific P removal rate of 4.5 mg P removed/g MLSS/day. Applying the same rationale to the 2001 data produced a specific P removal rate of 5.1 mg P removed/g MLSS/day. These data give a more accurate reflection of the relative performance of both systems as they compensate for differences in flow, loading and size of the plant. From this result it is clear that the 2001 microbial populations were more efficient from a nutrient removal standpoint than those present in the 1997 system despite the lower recycle rates and reduced zone capacity of the unaerated fraction. This improved performance under reduced HRT confirms findings from other studies with BNR systems, providing that the HRT is sufficient to support the growth of nitrifiers and PAB (Liu *et al.*, 1996).

Similar mass balance calculations were performed for NH_4-N (Table 5.4) and COD (Table 5.5) removal. Again, it is evident from Table 5.4 that a higher NH_4-N load (31,198 mg/day) was entering the 2001 pilot plant relative to the 1997 loading (21,672 mg/day). However, when the specific nitrification rates were determined, the 2001 plant had a higher nitrification rate than the 1997 design, with values of 7.6 and 5.1 mg NH_4-N removed/g MLSS/day being achieved, respectively. This result was very promising as one of the main concerns with the reduction in HRT was how these changes would affect nitrification, owing to the fact that nitrifiers are known to be especially sensitive to changes and have slower growth rates (Eckenfelder and Grau, 1992). It is clear that the new 2001 pilot-scale bioreactor design set-up did not impact negatively on nitrification. As mentioned previously denitrification was very satisfactory at all times during both trials.

Table 5.5 shows that the microbial population in the 2001 trial was, despite the shorter retention time, capable of removing COD at a faster rate than that observed in the 1997 trial, with values of 211 mg $COD_{removed}$/g MLSS/day

Table 5.3. Phosphate removal mass balance in the 1997 and 2001 trials.

	1997 pilot trial	2001 pilot trial
Forward flow (l/h)	30	21
Forward flow (l/day)	720	504
Influent P (mg/l)	26.7	57
Influent P load (mg/day)	19,234	28,728
Effluent P (mg/l)	0.5	17
Effluent P (mg/day)	360	8,568
P removed (mg/day)	18,864	20,160
Total MLSS in plant (g)	4,183 (5,260; 978)	3,944 (6,924; 1,341)
mg $P_{removed}$/g MLSS/day	4.5	5.1

Values in parentheses indicate upper and lower extremes.

Table 5.4. NH_4-N removal mass balance in the 1997 and 2001 trials.

	1997 pilot trial	2001 pilot trial
Forward flow (l/h)	30	21
Forward flow (l/day)	720	504
Influent N (mg/l)	30	62
Influent N load (mg/day)	21,672	31,198
Effluent N (mg/l)	0.3	2.2
Effluent N (mg/day)	216	1,114
N removed (mg/day)	21,456	30,084
Total MLSS in plant (g)	4,183	3,944
mg $N_{removed}$/g MLSS/day	5.1	7.6

Table 5.5. $COD_{soluble}$ removal mass balance in the 1997 and 2001 trials.

	1997 pilot trial	2001 pilot trial
Forward flow (l/h)	30	31
Forward flow (l/day)	720	504
Influent COD (mg/l)	1,008	1,757
Influent COD load (mg/day)	725,760	885,528
Effluent COD (mg/l)	43.3	102.7
Effluent COD (mg/day)	31,176	51,761
$COD_{removed}$ (mg/day)	694,584	833,767
Total MLSS in plant (g)	4,183 (5,260; 978)	3,944
mg $COD_{removed}$/g MLSS/day	166	211

Values in parentheses indicate upper and lower extremes.

and 166 mg $COD_{removed}$/g MLSS/day being attained, respectively.

5.4 Conclusions

- Over the peak period of the 2001 dairy season, the influent waste water contained elevated COD (1,697 mg/l), NH_4-N (61 mg/l) and P (49 mg/l), and the pilot plant was capable of achieving 96%, 97% and 74% removal of these parameters, respectively, over a sustained period.

- It is clear from the 2001 trial that the key to successful nutrient removal at Kerry will be to supply enough suitable C for both the removal of elevated N and P during the peak season. Biological P removal is capable of being sustained in the off-peak season, despite the colder weather conditions and lower organic loading to the plant.

- Using the 2001 system design, the process is capable of treating the elevated nitrogenous loading to the plant during the peak season and is capable of also performing reliably in the colder off-peak season.

- Reductions in the (i) size of the anaerobic and anoxic zones, relative to the total size of the mixed react zones, (ii) ratio of RAS to influent flow rate, and (iii) ratio of internal recycle flow rate relative to that of the influent flow rate do not negatively impact on the BNR process. In fact, the activity of the bacteria in terms of COD, NH_4-N and P removal was enhanced by these changes in operating strategy.

- The changes introduced in the pilot plant design of 2001 will reduce the cost of commissioning and designing a full-scale plant, as smaller anaerobic and anoxic tanks can be built and also lower recycle rates will reduce pumping costs.

5.5 Studies on Further Factors Affecting the EBPR Plant

Following the implementation of the new reactor configuration in late 2002, the pilot BPR plant operated well during the off-peak winter season (mid-October to mid-March) assuring efficient BPR where 85% P removal was achieved irrespective of variable pH and

temperature. During the month of March, Kerry Ingredients entered their peak production phase, which provided an ideal opportunity to monitor the response of the pilot plant to shock loading conditions that are generally associated with increased production. It was agreed to further shock the system by varying the COD, NH_4-N and PO_4-P loadings as follows: COD: >2,000 mg/l, NH_4-N: >60 mg/l and PO_4-P: >50 mg/l.

5.5.1 COD shock loading

Table 5.6 details the reactor performance prior to, during, and after COD shock loading. From Table 5.6 it is clear that there was no impact on effluent quality when the COD exceeds the value stipulated for shock loading (2,000 mg COD/l) as the final effluent COD values obtained during the COD shock period compare well with those obtained pre- and post-shock and satisfy the discharge. This result was expected as food processing waste waters are typically low in COD so an increase would be beneficial to the organisms responsible for BNR.

5.5.2 NH_4-N shock loading

Table 5.7 details the reactor performance prior to, during, and after NH_4-N shock loading. Table 5.7 shows that the shock loading period had a deleterious effect on process efficiency with respect to NH_3 oxidation. Prior to the shock loading period the pilot plant easily fulfilled the discharge limits imposed on Kerry Ingredients of 1 mg NH_4-N/l. During the shock loading period the final effluent NH_3 concentrations increased on average by a factor of 4.5, resulting in a non-compliant final effluent. It was pleasing to note that the shock loading period did not have any long-term effects as once the excessive NH_3 concentrations subsided the final effluent quickly returned to normal, thus satisfying the discharge limits.

5.5.3 PO_4-P shock loading

Table 5.8 details the reactor performance prior to, during, and after PO_4-P shock loading. According to the data presented in Table 5.8 the influent PO_4-P concentrations were quite variable and only exceeded the shock loading threshold once (9 April 2003). The data suggest that PO_4-

Table 5.6. COD shock loading.

Time period	Date	Influent COD (mg/l)	Effluent COD (mg/l)
Pre-shock	18 March 2003	1,865	81
	19 March 2003	1,840	62
	20 March 2003	1,820	83
Shock	27 March 2003	2,000	73
	28 March 2003	2,048	68
	31 March 2003	2,225	77
Post-shock	7 April 2003	1,846	64
	8 April 2003	1,818	60
	9 April 2003	1,630	68

Table 5.7. NH_4-N shock loading.

Time period	Date	Influent NH_4-N (mg/l)	Effluent NH_4-N (mg/l)
Pre-shock	18 March 2003	41.7	0.32
	19 March 2003	44.5	0.36
	20 March 2003	41.5	0.60
Shock	27 March 2003	69.0	1.00
	28 March 2003	69.5	1.80
	31 March 2003	74.0	3.00
Post-shock	7 April 2003	39.9	0.62
	8 April 2003	52.5	0.28
	9 April 2003	58.6	0.42

Table 5.8. PO_4-P shock loading.

Time period	Date	Influent PO_4-P (mg/l)	Effluent PO_4-P (mg/l)
Pre-shock	3 April 2003	41.5	2.2
	4 April 2003	38.1	1.4
	7 April 2003	39.0	5.0
Shock	8 April 2003	30.2	5.6
	9 April 2003	50.7	7.2
	10 April 2003	29.8	7.8
Post-shock	11 April 2003	28.8	3.9
	12 April 2003	34.5	3.1
	13 April 2003	45.6	2.6

P shock loading does not have a significant impact on BPR.

5.5.4 Variations in the pH of influent waste water

Since the amendments in reactor configuration in November 2002 the influent pH has demonstrated significant variability between 6.02 and 11.9. Figure 5.7 is a graphical representation of the effect of variable pH on COD, NH_4-N and PO_4-P removal by the reactor. From Fig. 5.7 it is clear that fluctuations in pH do not really have an appreciable effect on COD, NH_3 or PO_4 removal.

5.5.5 Increases in PAF

From September to November (Tables 5.9 and 5.10) the percentage of PAF waste water in the influent was increased to 100%, while the anaerobic HRT was maintained at 3.2 h. This period coincided with a reduction in production at the plant; thus, this parameter change did not result in an increase in the influent COD/P (value dropped from the previous phase from 43 to 37). Although the COD availability in the plant diminished, the PO_4 concentration in the raw waste water was still 37 mg/l (similar to the June, July and August waste water). Phosphorus removal was shown to increase significantly during this time, with effluent values averaging 10 mg/l. This was a significant improvement on previous effluent values obtained (26 mg P/l and 34 mg P/l, respectively).

5.6 Changes in Pilot-Scale Operational Parameters

Since the pilot trial had begun, there had been some design alterations to the full-scale plant. The original pilot trial design, described in the previous section, was based on the anaerobic and anoxic zones comprising 10% each of the total react volume of the plant. Therefore the aerobic zone formed 80% of the pilot plant. This was based on the assumption that the retrofit of the full-scale

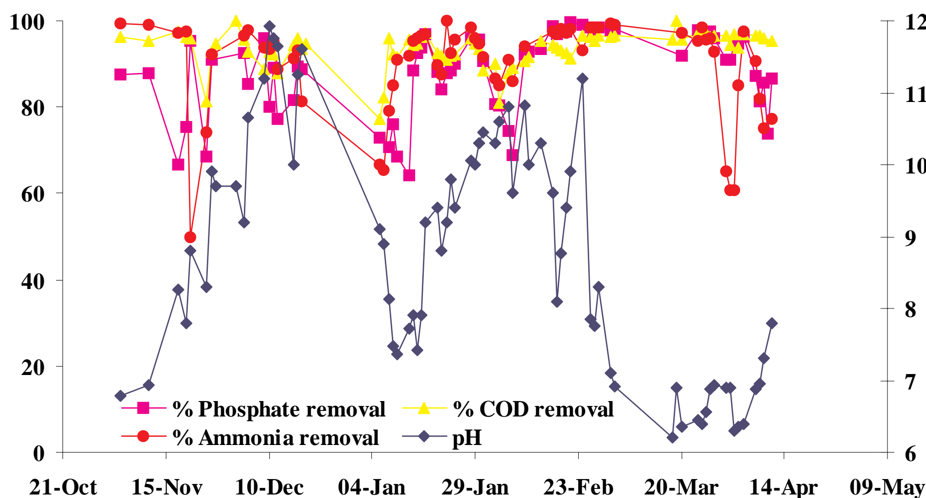


Figure 5.7. The influence of pH on process efficiency.

Table 5.9. Increases in PAF in early September[1].

Influent (mg/l)	Effluent $T_{0–24}$	Effluent $T_{24–48}$	Effluent $T_{48–72}$	Effluent $T_{120–144}$
COD- 1742	41	50 (2240)	41	43
NH_4-N- 58	0.28 (67)	0.28 (77)	0.18 (96)	0.34 (85)
PO_4-P- 19	4	6	5	5.5
NO_3-N-	0.8			

[1]COD/P Influent: 92. Anaerobic HRT: 3 h. MLSS: 5943.

Table 5.10. Increases in PAF from late September to early October[1].

Influent (mg/l)	Effluent $T_{0–24}$	Effluent $T_{24–48}$	Effluent $T_{48–72}$	Effluent $T_{120–144}$
COD- 1915	36 (2404)	50	79	43
NH_4-N- 58	0.28 (67)	0.28 (77)	0.18 (96)	0.34 (85)
PO_4-P- 19	6.08	28.7 (57)	10.6 (58.4)	4.6
NO_3-N-	0.85	0.8	0.9	0.9

[1]COD/P Influent: 44. Anaerobic HRT: 3 h. MLSS: 6726.

plant would involve constructing two new tanks to form the anaerobic and anoxic zones, and the three existing aeration basins (Tanks A, B and C) would form the aerobic fraction of the plant. However, the full-scale plant had undergone a retrofit to allow one of the existing aeration basins to become an anoxic zone. Consequently, this resulted in an increase in the relative size of the unaerated fraction of the plant (i.e. bigger anoxic zone), with a simultaneous reduction in the aerobic fraction. Therefore, to permit a realistic prediction of how the BNR process would perform in the new full-scale plant, it was necessary to retrofit the pilot plant accordingly. With this in mind, the anaerobic zone was designed to be 8% of the total volume, the anoxic zone was increased from the original value of 10% to 29%, and the aerobic fraction was reduced from 80% to 63%.

As there was evidence of a re-establishment of the BPR bacteria in the pilot plant, the anaerobic HRT was again reduced from 3 h to 2 h. The feed to the pilot plant still consisted of 100% PAF effluent (influent COD/P of 43:1). Under these new operating conditions (i.e. an increased unaerated fraction/reduced aerobic fraction) 80% P removal was achieved, with the influent P concentration of 28 mg P/l being reduced to 4.0 mg P/l in the effluent. This showed that the bacteria acclimated quickly to the changing environmental conditions in the reactor (Table 5.11). This level of P removal was very satisfactory considering that during this phase of operation the pH of the influent to the pilot plant increased significantly from 7.2 (Phase 8) to 9.45.

From late January to early March, the operating conditions remained unaltered (Table 5.12). The bacteria in the pilot plant adjusted to the higher pH prevalent in the incoming waste water, as was evident from the 93% P removal obtained during this period. This reflected a reduction in influent PO_4 from 30 mg P/l to an effluent value of 2.2 mg P/l. It is possible that some of the P removal being observed during the phases with high pH in the waste water may be the result of chemical precipitation rather than biological removal. Chemical precipitation of PO_4 is common at pH values >9. It appears that the main form of P removal during late November to early January may have been by chemical means, as the increase in P removal during this period was achieved while the anaerobic release and the anoxic uptake of PO_4 were lower during this phase than in the previous part of this study, September to early November, which had a lower overall P removal (73% P removal). However, from February to early March the improvement in both anaerobic release (2.7 mg P/l to 16.4 mg P/l in the anaerobic zones of Phases 9 and 10, respectively) and anoxic P uptake (24 mg P/l and 38 mg P/l in the anoxic zones of Phases 9 and 10, respectively) is evidence that biological P removal was responsible for the improvement in P removal.

Table 5.11. BPR operational parameters in early January[1].

Influent (mg/l)	Effluent $T_{0–24}$	Effluent $T_{24–48}$	Effluent $T_{48–72}$	Effluent $T_{120–144}$
COD- 2586	146 (2730)	90 (2430)	98 (3685)	113
NH_4-N- 41	1.84	1.68	1.45	1.9
PO_4-P- 46.6	3.3	2.9	2.2 (68.3)	3.2
NO_3-N-	0.8	0.6	1	2

[1]COD/P Influent: 56. Anaerobic HRT: 2 h. MLSS: 5462.

Table 5.12. BPR operational parameters in February to March[1].

Influent (mg/l)	Effluent $T_{0–72}$	Effluent $T_{72–96}$	Effluent $T_{96–120}$	Effluent $T_{192–216}$
COD- 2186	91 (2538)	87	86 (2405)	67
NH_4-N- 33	0.45 (60)	0.7	0.38	0.34
PO_4-P- 32	0.46	0.92	0.46	0.54
NO_3-N-				

[1]COD/P Influent: 69. Anaerobic HRT: 2 h. MLSS: 6873.

5.7 The Acidogenic Reactor

Work was also performed which involved assessing the performance of the acidogenic reactor system under various hydraulic loading rates. It is clear that one of critical control points with regard to the operation of the BNR process is the quality of the influent waste water. The biology of the organisms responsible for BNR has been extensively studied over the past few decades. The BNR process in operation at the plant is based on the typical A^2O configuration, i.e. the influent waste water initially encounters the anaerobic zone, followed by exposure to anoxic conditions and finally the waste water is subjected to aerobic conditions. The function of the anaerobic zone is generally accepted to be selective enrichment. As the biomass is recycled through the system it passes through the various zones where O_2 may or may not be present. Therefore, during residence in the anaerobic zone, organisms of facultative nature (BNR organisms) have a competitive advantage over their strictly aerobic counterparts. This is the main reason for inclusion of the anaerobic zone at the front end of the system. The competitive advantage of facultative organisms is displayed as follows: during residence in the anaerobic zone the facultative organisms are capable of sequestering the available substrate to drive the BNR process, in particular the BPR process, while strictly aerobic organisms are incapable of substrate accumulation. During residence in subsequent zones the strict aerobes do not have access to substrate and thus over time will be removed from the system.

In theory, this process is very successful but in order to attain and maintain successful BNR the quality of the incoming waste water is of paramount importance. Typically food processing waste waters are rich in P (10–50 mg/l) and N (20–70 mg/l) with COD concentrations ranging from 1,500 to 2,500 mg/l. The COD demands of a BNR system are generally quite high, which means that the influent COD concentration and quality are one of the most important factors in assuring successful BNR.

During the initial investigation of the waste water from the plant it was determined that, even though the P and N concentrations were high during the peak production months, the COD reflected this situation and was considered sufficient to support BNR. As stated previously the key to BPR is anaerobic sequestration of substrate to confer the competitive advantage upon the organisms responsible. If the waste water was passed to the anaerobic zone without acidogenic pre-treatment the BPR organisms may not be able to accumulate sufficient substrate to drive BPR. More importantly, the organic loading would pass through the anaerobic zone to the subsequent anoxic and aerobic zones. This occurrence would lead to competition for available substrate between the strictly aerobic and facultative organisms during oxygenation, ultimately resulting in depletion of the P removal capability of the unit. As a solution to this

potential problem a side-stream acidogenic rector was put in place to pre-acidify the influent waste water, which in turn conferred the competitive advantage on facultative organisms thus assuring efficient BPR. The requirement for pre-acidification is explained by the biology of the BNR organisms present in the system. Facultative organisms responsible for BPR under anaerobic conditions will accumulate COD to drive the process if it is present in a particular form, i.e. VFAs. There are many references available to substantiate the fact that pre-fermentation of the influent waste water serves to improve the BNR capability of the process. The acidogenic process is essentially a truncated anaerobic digestion process. During acidogenesis complex organic molecules (e.g. sugars) are broken down into their constituent VFAs which are the ideal substrates for BPR organisms. There are a number of key operation parameters associated with the acidogenic process with HRT being the most important.

Acidification of influent waste water is essentially a balancing act. It is important to allow sufficient time for complex organic molecule breakdown into VFAs while at the same time preventing progression of the process to complete anaerobic digestion. The primary reason for this requirement is maintenance of COD strength. An efficient acidification reactor should ideally ferment the influent waste water (VFA production) with a minimum of COD consumption.

In order to investigate this process fully with respect to the rector configuration at the plant, it was decided to operate the pre-acidification reactor at a number of HRTs as follows: (a) 14 h, (b) 24 h, (c) 33 h and (d) 47 h. The overall result of this trial was that irrespective of HRT amendment, VFA production capacity of the acidification reactor remained unchanged, increasing the VFA content of the waste water on average by 20%.

5.8 Varying Internal Recycle Flow Rates

The effect of varying internal recycle flow rate on final discharge values was also analysed. The focus here centred on internal recycle flow rates with a view to the optimisation of denitrification. As previously stated, the Kerry Ingredients pilot BNR plant was initiated to investigate both N and P removal from the influent waste water. This was accomplished through modification of the standard AO waste-water treatment plant configuration (primarily for P and organic contaminant removal) with the inclusion of an anoxic zone with the express intention of denitrification establishment within the reactor. The organisms responsible for nitrification and denitrification have been well studied and applied to waste-water treatment for some time now. The biology of the nitrification and denitrification process is quite simple as described below.

Conventional biological N removal is achieved by means of nitrification followed by denitrification. Nitrification is an autotrophic process where the sole requirements are the presence of the relevant organisms, NH_3-N and O_2. Nitrification can be further subdivided into nitritation (NH_3 conversion to nitrite (NO_2)) and nitration (NO_2 conversion to NO_3). However, denitrification is catalysed by heterotrophic bacteria (COD requiring) where the NO_3 produced during nitrification is reduced to di-nitrogen gas. In a waste-water treatment plant where BNR is sought, various design considerations apply. In order to accommodate both N and P removal simultaneously on the Kerry Ingredients site the $A^2 0$ process design was applied. This design consists of a front-end anaerobic zone for selective enrichment of BPR organisms, followed by an anoxic zone where denitrification occurs and on occasion organisms can develop with the ability to perform BPR under anoxic conditions. The final zone is an aerobic zone where nitrification and BPR occurs. In order to encourage denitrification, a recycle from the aerobic zone to the anoxic zone has to be included where nitrified (NO_3 containing) waste water is returned to the aerobic zone to stimulate denitrification. Control of nitrified waste-water recycle is extremely important, as there is a significant cost factor associated with waste-water pumping. Therefore, during this study, consideration had to be given to internal recycle optimisation. Throughout the course of the trial three separate internal recycle regimes were applied as follows:

1. 1*Q* – the internal recycle was equivalent to the influent flow rate

2. 2*Q* – the internal recycle was twice the influent flow rate, and

3. 3*Q* – the internal recycle was three times the influent flow rate.

In order to determine the influence of varied internal recycle rates on process operation, the percentage P, NH_3 and NO_3 removal rates were assessed (Table 5.13).

Table 5.13. The influence of internal recycle on process operation.

Q	% P removal	% NH_4-N removal	% NO_3-N removal
1	61	96	50
2	70	96	40
3	77	98	32

From the data obtained it is clear that, firstly, the P removal appeared to improve in response to increased recycle rate. This improvement was more likely due to an improvement in COD source and cannot be directly attributed to increased recycle rate. Secondly, the NH_3 removal rate also increased slightly which was primarily due to the periodic dips in NH_3 loading over the trial period. Finally, and most importantly, NO_3 removal decreased in response to the change in recycle rate that prompted a return to 1*Q*.

This final observation is particularly significant. The ability of the reactor to denitrify the NO_3 present following aerobic operation at an internal recycle rate of 1*Q* could result in significant energy savings due to the decrease in pumping (waste-water recycle) costs. This result was expected, as the influent NH_3 concentration did not fluctuate significantly apart from the changeover from low to peak season. During this changeover period the reactor responded negatively and required on average a 2-week period to acclimate to the increased NH_3-loading rate. It is suggested that this occurrence could be circumvented by gradually increasing the NH_3-loading rate artificially over a 2- to 3-week time period in preparation for peak operation.

5.9 Conclusions

Thus, it is clear from these studies on the pilot-scale BPR system that the plant is quite robust and capable of operating following exposure to a large variety of different operational parameters such as changes in COD, NH_3 and P influent levels. It is also clear that internal recycle flow rates are also important in determining the rate of not only P removal from the waste streams but also the level of NO_3 removal.

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