Aerobic Granular Systems: the Treatment of Nitrogen Deficient Wastewater

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ABSTRACT

This Masters thesis demonstrated for the first time that it is possible to generate aerobic granules using a nitrogen deficient wastewater; and that such systems utilise nitrogen fixation for sustaining balanced growth. Furthermore, the performance of nitrogen fixing granular systems was shown to be comparable to conventional aerobic granular systems: they exhibited a high level of carbon removal and excellent biomass settleability.

Using a specially developed image characterisation technique, granule parameters were analysed to investigate the formation of nitrogen-fixing granules and subsequently to compare the effects of turbulence on these nitrogen fixing granules; a nitrogen supplemented system was utilised as a control. Sequencing batch reactors (SBRs) with a short (1 minute) settling phase were used for the experiments. The length of the settle phase was based on the results of a preliminary study which showed that granules grown with a 1-minute settling time are significantly larger (and more irregular) than granules grown in reactors with longer settling times.

Granules were generated in both the nitrogen deficient system and the control. The difference between the granules grown under nitrogen-deficient and nitrogen-rich conditions was evident through image analysis revealing differences in granule size and structure. Those granules grown without nitrogen supplementation were denser and larger than those grown with supplementation. It is proposed that the difference in morphology is due to the function of nitrogen-fixing bacteria in the nitrogen deficient system. These bacteria utilise the nitrogenase enzyme and so prefer an environment with low oxygen concentration. It is proposed that nitrogen fixers proliferate inside the granule due to the oxygen concentration gradients created through diffusion limitations.

The effect of turbulence levels on the formation of nitrogen-fixing granules was pronounced. The comparison of high and moderate aeration/turbulence reactors found that granules grown under the less turbulent regimes were more filamentous, irregular

and had slower settling velocities than those grown under greater turbulence. Without a minimum turbulence threshold granules were not generated and a biofilm became the dominant microbiological form within the reactor.

Based on the results of this work, it is proposed that an SBR with a short settling phase and high turbulence could be employed to develop nitrogen-fixing granules for the treatment of nitrogen deficient wastewater. The primary benefits for such a system are good sludge settleability and removal of the need to supplement nitrogen for growth.

Notice of Publications

Some information and research from this thesis has been published or submitted.

1. Tan L.B.M., Gapes D.J., Pratt S. (2004) Image Analysis of Aerobic Granulation in Biological Wastewater Treatment Systems. Proceedings from the 2004 APCSEET Conference. Wellington.

   - information from Chapter 4 and Chapter 5

2. Pratt S., Tan M., Gapes D., Shilton S. (2006) Development and examination of a granular nitrogen-fixing wastewater treatment system. Journal of Process Biochemistry. *Submitted.*

   - information from Chapter 6

3. Tan L.B.M., Gapes D.J., Pratt S. (2005) Growing Granules to Treat Nitrogen Deficient Waste. Poster in the MacDiarmid Young Scientist of the Year Awards 2005.

   - information from Chapter 4 and Chapter 5

Chapter 1: Introduction

Wastewater treatment technologies have continued to become increasingly important for both municipal and industrial facilities. The removal of solids, carbon, and nutrients are basic requirements for treatment systems. Microbial systems, in combination with separation processes, have been used successfully as a means of removing all three components from wastewater.

Carbon within wastewater is used by microorganisms for growth and energy. For continued population growth these microorganisms require nitrogen, phosphorus and other nutrients. Generally these nutrients are in over-supply and microbial uptake and conversion is welcomed, resulting in removal of environmentally and ecologically harmful compounds. But some wastewaters are nutrient deficient. Traditionally, treatment of such wastewaters has required nutrient supplementation which incurs a cumulative upkeep cost as well as the problems associated with under and over addition of these nutrients. More recently, the potential for treatment by nutrient-fixing (namely nitrogen-fixing) organisms has being recognised (Clark *et al.*, 1997; Gapes *et al.*, 1999; Dennis *et al.*, 2004).

All microbial treatment systems require separation of the solid and liquid phases prior to final effluent discharge. In particular, activated sludge systems rely on gravity settling to produce high quality treatment performance. Systems with poor settleability have problems separating these two phases. Nitrogen-deficient activated sludge systems have been found to have high levels of filamentous bacteria and/or extracellular polymeric material (Andreasen *et al.*, 1999; Thompson and Forster 2003, Dennis *et al.*, 2004) which can result in sludge bulking and thus results in poor settling properties within such systems. Solutions to this problem would provide the novel nitrogen-fixation based treatment technology with the potential for significantly broader application for the treatment of nitrogen deficient wastewaters.

Aerobic granular activated sludge technology has been identified as a promising new technology. This process has been found to form finite, well defined, aggregates comprised of cellular biomass and extracellular constituents. The aggregates themselves have been found to have superior settling characteristics whilst maintaining good treatment of influent carbonaceous material.

It has been found that, for the development of granules within aerobic reactors, sequencing batch reactors (SBR) are preferential (Liu and Tay 2004a). The SBR systems are run under a discontinuous flow regime with a set period of time allocated for each stage of the complete reaction cycle. This discontinuous regime has been found to have advantages, with feast to famine feeding schedules and periods of high turbulence balanced with periods of quiescence.

A review of the literature found that no publications existed on nitrogen fixing granular systems. While publications existed on each topic separately it was unknown if the two technologies could be successfully integrated into a single system. The core of this challenge was the conflict between requirement for low oxygen concentrations for the functionality of the nitrogen fixing enzyme, nitrogenase, and the granule associated requirement of high turbulence, which in the majority of cases is in the form of sparging air into the reactors. However, some preliminary experimentation led to the hypothesis that nitrogen fixing granular systems could be a viable technological opportunity.

Literature alluded to the ability of shortened settling times to aid in the selection of faster settling particles which can serve as a starting point for granular growth. Similarly the effect of turbulence on regular systems has been noted. However, the effect of increased turbulence on systems where nitrogen fixation is necessary has not been investigated.

The central aim of this thesis was the generation of an aerobic granular activated sludge system, that would be capable of effective treatment of nitrogen deficient wastewater without the supplemental addition of nitrogenous compounds. Specifically, the objectives of this thesis were to investigate:

1. The effect of extreme nitrogen deficiency, in a carbon containing wastewater, on granulation.

2. The effect of settling time on selection and granule characteristics.

3. The effect of turbulence on granules grown under nitrogen deficient conditions.

These objectives, are further detailed within the research questions posed following the Literature Review.

Chapter 2: Literature Review

2.1 Introduction

Environmental policies for both industrial and municipal wastewater continue to become stricter. Continual advancement in wastewater treatment technology is required to meet the increasingly stringent threshold limitations for these wastes.

One such advancement is the development of nitrogen-fixing activated sludge systems, whereby micro-organisms capable of converting atmospheric nitrogen into biochemically available forms are harnessed to provide a nitrogen deficient system with enough nitrogen for growth to occur. Both nitrogen fixing and conventional activated sludge technologies have similar processes. Through aerobic biological reaction, biomass aggregates form, usually in the form of bioflocs (clusters of microbial cells and colonials that have agglomerated together), and then the unsettled portion is discharged within the effluent. Unfortunately, the potential for relatively poor sludge settleability of the nitrogen-deficient systems is an issue for full-scale implementation (Andreasen *et al.*, 1999; Thompson and Forster 2003, Dennis *et al.*, 2004).

An approach for ensuring effective separation of solids from treated effluent is to immobilize biomass in the form of microbial granules. Compared to bioflocs, granules have high density, strong microbial structure and importantly good settleability. This literature review was carried out to investigate how granulation could potentially advantage nitrogen fixing wastewater treatment systems. As this thesis involved an attempt to generate hybrid nitrogen fixing aerobic granular technology, both nitrogen fixation and aerobic granular systems were reviewed.

2.2 Nitrogen Fixation

Nitrogen fixation is the reduction of atmospheric nitrogen to ammonium, catalysed by the nitrogenase enzyme, as shown by the following reaction:

$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$ (Dixon and Kahn, 2004)

The process is an anaerobic or microaerophilic process utilised by aerobic, facultatively anaerobic or anaerobic bacteria (Mulder and Brotonegoro, 1974; Starr *et al.*, 1981). The component proteins of nitrogenase are extremely oxygen sensitive and thus aerobic bacteria have been found to possess varying mechanisms to survive oxygen inhibition, including respiratory protection, enzyme conformational protection and alginate or slime formation (Postgate, 1974; Oelze, 2000; Sabra *et al.*, 2000). Such mechanisms allow two apparently paradoxical processes to occur together within the same cell, namely aerobic respiratory metabolism, coupled with anaerobic nitrogenase activity.

2.2.1 Nitrogen Fixing Microorganisms

A number of different organisms and groups have been found to have nitrogen-fixing potential. The conditions under which these bacteria are able to operate also differ which means that different populations can be utilised to acquire nitrogen even under varied conditions. A list of these bacteria can be found in Table 2.1. This emphasises the different conditions that can facilitate nitrogen fixation. Within similar nitrogen deficient studies (Gapes *et al.*, 1999; Dennis *et al.*, 2004) the bacteria of interest have been those that can survive under aerobic and microaerophilic conditions.

Table 2.1 Bacteria known to fix nitrogen under different conditions (Baliss et al., 1996; Chen et al., 1984; Gapes et al., 1999; Kargi and Özmihçi, 2002a, 2002b, 2004)

Aerobic	Microaerophilic	Anaerobic
Azotobacter chroococcum	Enterobacteriaceae	Vibrioaceae
Azotobacter vinelandii	Methylococcaceae	*Bacillus*
Azotomonas	Pseudomonadaceae	*Klebsiella*
Azotococcus	Rhizobiaceae	*Rhodopseudomonas*
Biejerinckia spp	Beggiatoaceae	*Clostridia*
Derxia spp	*Azospirillum spp*	
Escherichia Coli	*Xanthobacter spp*	
	Mycoplana spp	
	Campylobacter sputorum	

2.2.2 Application for Nitrogen Fixation: Treatment of Nitrogen Deficient Wastes.

Nitrogen fixation has been found to be a significant component of the nitrogen cycle as it is the primary means for atmospheric nitrogen to be converted into biologically available forms (Brock *et al.*, 1984). Its uses within industrial processes have only just begun to be explored.

For the treatment of a nitrogen-deficient wastewater the nitrogen fixation process becomes important as it allows a system to self-regulate the concentration of nitrogenous compounds. This results in lower concentrations of soluble nitrogen within the effluent streams of system where fixation occurs than supplemented systems (Dennis *et al.*, 2004; Gapes *et al.*, 1999; Slade *et al.*, 2003 and 2004a). Two relevant industrial applications that have been investigated so far are that of the treatment of olive-milling (Baliss *et al.*, 1996; Ehaliotis *et al.*, 1999) and pulp and paper mill wastes (Dennis *et al.*, 2004; Gapes *et al.*, 1999; Slade *et al.*, 2003 and 2004a). The case of treatment of pulp and paper wastes is reviewed further.

Within the thermomechanical pulping wastewater used in the study by Dennis *et al.* (2004), the ratio of available carbon to nitrogen (measured as the BOD:N ratio) was found to be 100:0.8. This represents a substantial nitrogen deficiency; Möbius (1991) found that a maximum BOD:N ratio of 100:3.5 was required in order for optimal biomass growth to occur. Typically the nitrogen within this wastewater would be supplemented in the form of urea (Kargi and Özmihçi, 2002a, 2002b & 2004; Thompson and Forster, 2003) to reach a concentration to enable sufficient growth to occur. If the level of supplementation was too low, then optimal growth would not occur; if the level were too high, then elevated levels of soluble nitrogen would pass through the reactor increasing effluent nitrogen concentrations (Dennis *et al.*, 2004; Gapes *et al.*, 1999; Kargi and Özmihçi, 2002a; Slade *et al.*, 2003 and 2004a). Alternatively, a nitrogen fixation technology could be employed. Extensive testing has shown the inherent technology to be both robust and manageable and its elimination of nutrient addition has been noted to save between 25 and 35 % of operating costs (Slade *et al.*, 2003 & 2004b). Clark *et al.* (1997) investigated the treatment of pulp and paper wastewater occurring in aerated stabilisation basins where nitrogen fixation was known to occur. It was found that 600 kg/d of nitrogen was being fixed for microbial growth within the pond system. For this nitrogen requirement to be supplemented in the form of urea it would represent around 470 tonnes each year. This highlights some of the savings made through harnessing nitrogen fixation.

One issue that nitrogen-deficient activated sludge systems have is that of poor sludge quality. Frequently these systems are plagued with sludge bulking and poor settling solids through high proportions of highly filamentous bacteria (Andreasen *et al.*, 1999, Thompson and Forster, 2003; Slade *et al.*, 2004a). Despite this problem, nitrogen-fixing systems have been shown to effectively remove both the soluble and suspended contaminants from wastewater. Further details on sludge bulking are detailed within the flowing sludge quality section.

2.2.3 Limitations of Nitrogen Fixing Systems

2.2.3.1 Treatment Performance

A direct comparison between nitrogen supplemented and nitrogen deficient laboratory systems treating bleached kraft mill effluent (Gapes *et al.*, 1997) found that the nitrogen fixing system removed carbon at slightly lower efficiencies than the nitrogen supplemented system. However, the lower efficiencies were off-set by the fact that the concentration of ammonia within the nitrogen fixing effluent was found to be significantly lower (0.5 - 0.7 mg/L) than the nitrogen supplemented reactor (2.1 - 4.4 mg/L). This highlights one of the major advantages of nitrogen fixing systems, in that the cells regulate their nitrogen uptake through biochemical fixation, taking in only as much as they require. The soluble nitrogen concentrations are thus consistently extremely low, a very difficult task to achieve via controlled supplemental addition in full-scale treatment plants.

2.2.3.2 Sludge Quality

Sludge bulking is major indicator that a system has poor sludge quality. Bulking occurs when filamentous bacteria become dominant within a system and the branching filaments cause the biomass to clump together and impedes its settlement within the reactor.

A number of studies have been carried out to determine the difference between systems run under both nitrogen supplementation and nitrogen deficient conditions. The quality of sludge was found to be a common benchmark within many of these studies and emphasises the problems that may arise through bulking.

The quality of sludge is generally determined through the key parameter of the rate at which particles settle within a system. The sludge volume index, SVI, is the standard measure and is determined by the volume to weight ratio after the system biomass has settled for a period of time (for a more detailed description see *Chapter 3 Section 3.7.9*). Higher SVI values are indicative of systems that have poor settleability and thus their effluents tend to have correspondingly higher levels of solids.

Sheker *et al.* (1993) initially ran reactors in nitrogen deficient mode and then added nitrogen to the feed regime. This resulted in a major decrease in the SVI as it dropped from 550 down to 125 mL/g within 5 days of supplementation. Effluent from this system was found to be highly turbid following addition, due to high growth rates for dispersed bacteria.

The effect of carbon:nitrogen ratio within the feed has been investigated. It was found that altering this ratio also had a significant effect on the bulking nature of the sludge as is shown in Table 2.2 (Peng *et al.*, 2003). As the amount of nitrogen added to the system decreased to severely deficient levels, the SVI increased significantly and the biomass concentration within the reactor was unable to be maintained at 2.0 g/L.

Table 2.2 Effect of carbon:nitrogen ratios within the feed on reactor characteristics (Peng *et al.*, 2003).

BOD:N ratio	Sludge Volume Index (SVI) (mL/g)	Suspended Solids (MLSS) (g/L)
100:5	40	1.9 - 2.1
100:4	70	1.9 - 2.1
100:3	228	1.9 - 2.1
100:2	250	2.0
100:0.94	458	1.3

Kargi and Özmihçi (2002a & 2002b) found that systems where *Azotobacter* was present had an increased carbon removal efficiency and effluent carbon concentration. Kargi and Özmihçi (2002b) found that in order to achieve 90 % removal efficiencies within *Azotobacter* supplemented systems, the loading rate must be below 50 kgCOD/m^3/h.

Operating at high BOD:N ratios have also been found to have adverse effects on some activated sludge systems with bulking and filamentous growth causing poor settling of particles and high sludge volume indices SVI (Andreasen *et al.*, 1999; Peng *et al.*, 2003; Thompson and Forster 2003; Sheker *et al.*, 1993).

Dennis *et al.* (2004) found that within nitrogen fixing systems the SVI improved significantly when the apparatus used to grow the microbial populations was changed from a continuously stirred tank reactor (with an average SVI of 600 mL/g) to an SBR (average SVI 200 mL/g).

Clearly, poor sludge quality appears to be an intrinsic feature of nitrogen deficient systems and interaction with good settling granular technology is hoped to be beneficial. The use of SBR for generating nitrogen-fixing biomass represents a definite opportunity in improving sludge settleability and this has immediate and obvious parallels to the development of granular sludge.

2.2.4 *Affinity for Oxygen*

Slade *et al.* (2003) investigated the effect the dissolved oxygen concentration level had on an aerobic nitrogen fixing system. Three dissolved oxygen levels were investigated: conventional 30 % saturation, low 14 % saturation, and very low 5 % saturation. This study found that as the oxygen concentration decreased, the amount of solids within the bioreactor increased, reflecting a decrease in the sludge volume index (thus reduced bulking effect) as is shown in Table 2.3. Due to the decrease in oxygen concentration, greater levels of nitrogen fixation were able to occur, and this is reflected in the 'decreased' nitrogen removal efficiency found within the system when it was operated at low DO.

Table 2.3 Effect of Dissolved Oxygen concentration on treatment and reactor characteristics (Slade *et al.*, 2003)

	Convential 30 %	**Low 15 %**	**Very Low 5 %**
Dissolved Oxygen (mgO_2/L)	2.2	1.0	0.3
SVI (mL/g)	286	201	184
TSS (mg/L)	3535	4973	5280
COD_{tot} removal %	82	87	87
TKN removal %	71	54	24

2.3 Aerobic Granulation Technology

As bacteria and other micro organisms consume substrates and grow, they tend to aggregate together to form various types of macrostructures. Under various selection pressures, these aggregate forms can develop further to form a compact and dense body, which has been defined as a granule.

Aerobic granules are desirable for their ability to treat industrial and municipal wastewater and remove both organic carbon and nutrients from effluent streams (Dulekgurgen *et al.*, 2003; Yang *et al.*, 2004; de Kreuk *et al.*, 2005). Granules have high settling velocities which lead to good solid-liquid separation and high biomass retention times. Aerobic systems have been shown to have the propensity to treat wastewater with high loading rates and cope with variable influent concentrations.

Most studies have used sequencing batch reactor (SBR) systems to generate granules (Morgenroth *et al.*, 1997; Beun *et al.*, 1999; Tay *et al.*, 2001a, 2002, 2004a,b; Jiang *et al.*, 2002; Moy *et al.*, 2002; McSwain *et al.*, 2004; Schwarzenbeck *et al.*, 2004). The settling and decant stages have been found to have significant impact on the formation process. In contrast to continuously fed systems, SBR systems have varying substrate concentrations as the cycle advances through its sequences. This has the advantage of varying the penetration depth of substrate, through concentration gradients, into the granule potentially creating a feast and famine regime which has been found to enhance growth (Bossier and Verstraete, 1996). Figure 2.1 shows the sequences that occur with the SBR as well as the relative substrate and dissolved oxygen concentrations within the reactor during these periods.

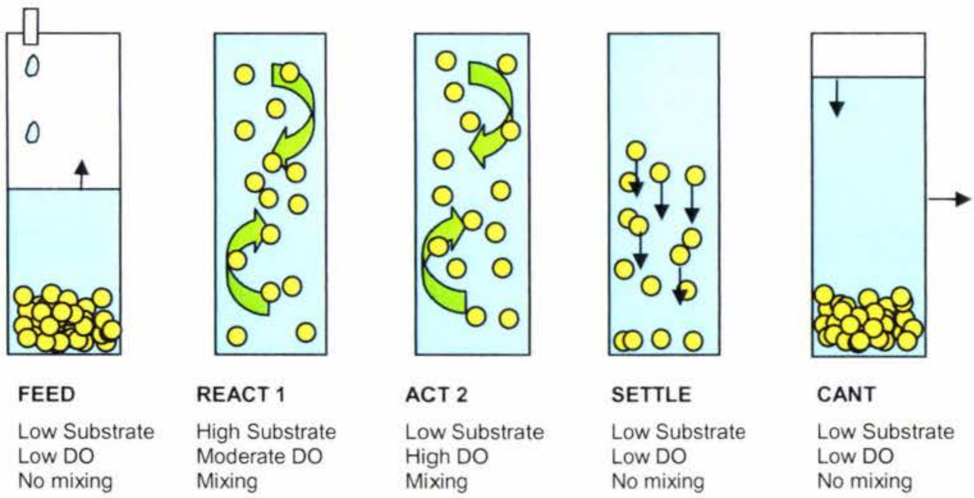


Figure 2.1 Schedule of Sequences within the Reactor

2.3.1 The Formation of Aerobic Granules

Figure 2.2 shows a granulation mechanism as proposed by Beun *et al.*, (1999). Initially, filamentous bacteria, found within the inoculum, bind together and enmesh other bacteria and particulate material, forming the initial-stage granule. Hydrodynamic forces within the system cause attrition of these filamentous off-shoots. Bacterial micro-colonies form within the granule. As the granule continues to grow, the central structure of the granule begins to weaken, until the granule breaks apart. The micro-colonies however, are stable enough to act as cores for new granular growth.

Reactor inoculum, whether it be granules, flocs or suspended cells, was found to alter the mechanism in which granules were formed with larger particles taking less time to morphologically alter to become more granular characteristically (Beun *et al.*, 1999).

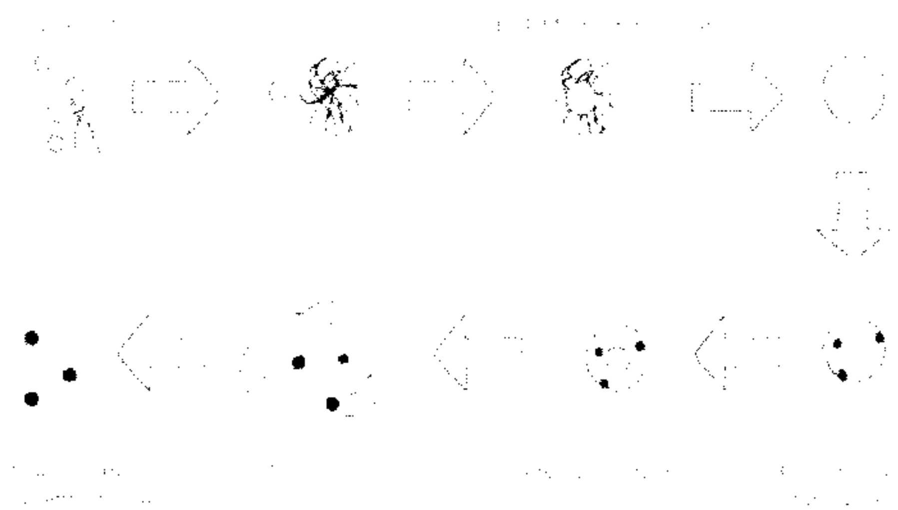


Figure 2.2 Proposed mechanism of granulation after the start up of a SBR with a short settling time (Beun *et al.*, 1999).

Growth within a granular system can occur via increased numbers of free-swimming bacteria, small flocs or on the surface of pre-existing granules. This implies that the hydraulic retention time (HRT) will effect granule formation. Reduced retention times result in higher levels of washout of the poor settling, non granular particles (such as the free-swimming bacteria and flocs) within the reactor (Morgenroth *et al.*, 1997; Beun *et al.*, 1999; Jang *et al.*, 2004; Pan *et al.*, 2004). Pan *et al.* (2004) found that at low HRT (1 h) all the biomass within the system was washed out resulting in reactor failure. At a long HRT (24 h) the granule population was gradually substituted by bioflocs. Short HRT were also found to have decreased sludge production but the size of granules was found to be larger than at longer HRT.

The ideal temperature and pH conditions for aerobic granulation have not been investigated. Liu and Tay (2004) suggested that these two parameters are not as important for aerobic systems as they are for anaerobic systems. Generally aerobic systems have been run between 20 and 25 °C which is lower than for their anaerobic counterparts.

2.3.2 Factors Affecting Aerobic Granulation

2.3.2.1 Settling Time

Aerobic granular biomass has been found to have faster settling velocities and higher densities than flocculent biomass. As such, reducing the period of time allowed for solid-liquid separation can be used for preferential selection of granules (Beun *et al.*, 2002; Wang *et al.*, 2004). Sequencing batch reactors (SBR), which allow manipulation of settling and decant stages, are therefore ideal for granule development (eg. Morgenroth *et al.*, 1997; Beun *et al.*, 1999; Tay *et al.*, 2001a, 2002, 2004a,b; Jiang *et al.*, 2002; Moy *et al.*, 2002; McSwain *et al.*, 2004; Schwarzenbeck *et al.*, 2004).

The effect of settling time on the development of aerobic granules has been studied (McSwain *et al.*, 2004; Qin *et al.*, 2004). Only when the settling time of the SBR was shorter than 5 minutes would the suspended sludge be washed out of the system and granules become dominant within the system. In systems with longer settling times both flocs and granules were found simultaneously with neither form becoming predominant.

To help select for granules with faster settling velocities, reactors with high height to diameter (H/D) ratios have been used (Beun *et al.*, 1999). Particles with poor settling qualities are more likely to be washed out due to both the increased settling distance and the increased time required for particles to settle below reactor decant line, prior to effluent removal commencing. These reactors also have the added advantage of having a smaller reactor footprint.

2.3.2.2 Turbulence

Turbulence within a reactor has been found to have significant influence on formation of granules. Tay *et al.* (2001a,b) and Liu *et al.* (2003b) found that the turbulence, such as that caused by aeration, determines whether granulation will occur. At low superficial upflow air velocities of less than or equal to 0.8 cm/s no granules formed and fluffy flocs were dominant within the reactor. As the air velocity increased to 2.5 cm/s regular-shaped spherical granules became dominant.

The roundness of the granules was also found to be proportional to the superficial air velocity within the reactor (Liu *et al.*, 2003b). This smoothing of the granule surface was found to be caused by high gas velocities detaching filamentous outgrowth, which are washed out with the effluent (Beun *et al.*, 1999).

The upflow pattern in liquid and air column reactors was found to create a consistent circular flow dynamic within the reactor. This consistent shear is capable of creating regularly shaped granules. However, in reactors mixed with stirrers or impellers, areas of localised hydrodynamic forces are created. This dispersed flow regime means only irregularly shaped flocs are able to form (Liu and Tay, 2002). This shows that for regularly shaped granules to develop, shear forces within a system must be uniform.

Shown in Table 2.4 are the effects of increased turbulence on important characteristics within granular systems. As the applied specific upflow air velocity increased, the overall quality of granules within the system improved. Granules were denser and settled better whilst having a higher biomass concentration. Of potential importance to nitrogen-fixing granular systems it appears that increased extracellular polysaccharide production can also be stimulated with increased turbulence.

Table 2.4 Effect of increased Turbulence on system characteristics ["+" indicates an increase with increased turbulence, "-"indicates a decrease] (Tay *et al.*, 2001b)

Characteristic	Result
Biomass Concentration	+
Sludge Volume Index	-
Specific Gravity	+
Extracellular Polysaccharide Production	+
Specific Oxygen Uptake Rate	+

The superficial gas velocity used to create the upflow effect with the reactor often determines the dissolved oxygen within a reactor. In systems where mixing is desired during an anaerobic stage of a cycle the oxygen levels can be controlled using alternative gases such as argon and nitrogen (Lemos *et al.*, 2003; Arrojo *et al.*, 2004).

2.3.2.3 Substrate Composition and Loading

Most studies on aerobic granules have utilised a synthetic feed source with acetate often used as the carbon source (Tijhuis *et al.*, 1994; Beun *et al.*, 2001; Etterer and Wilderer, 2001; Tay *et al.*, 2001a; Beun *et al.*, 2002; Dulekgurgen *et al.*, 2003; Jang *et al.*, 2003; Liu *et al.*, 2003b; Toh *et al.*, 2003; Qin *et al.*, 2004). Other carbon sources include glucose (Etterer and Wilderer, 2001; Tay *et al.*, 2001a; Yu *et al.*, 2001; Tay *et al.*, 2004a,b; Wang *et al.*, 2004); ethanol (Beun *et al.*, 1999; Yang *et al.*, 2003 and Arrojo *et al.*, 2004); while molasses mixed with tap water was used by Morgenroth *et al.*, (1997), and Heijnen *et al.*, (1992) used effluent from an anaerobic reactor (a feed that is high in volatile fatty acids).

Tay *et al.*, (2001a) and Moy *et al.*, (2002) compared glucose and acetate feed solutions. It was found that the granules formed by these feeds were structurally different. Acetate fed granules tended to be smoother with no presence of filamentous bacteria, while glucose fed granules had a significant filamentous bacterial population resulting in a fluffy outer surface. Both types of granules had a round-shaped outer structure although the glucose-fed granules were both larger and denser than those fed on acetate. The removal of organic carbon, however, was insignificantly higher in the acetate (98 %) reactor than the glucose reactor (97 %). These studies show that the morphology of granules varied depending on the carbon source used. Due to the filamentous nature of bacteria that were found within systems where glucose was the carbon feed, acetate is a preferred feed media for enhancing the settleability of the granule population.

There have been few studies on granulation systems that have used industrially sourced wastewater (Arrojo *et al.*, 2004; Schwarzenbeck *et al.*, 2004; Su and Yu

2005). However it has been speculated that the colloidal material in real wastewater could be adsorbed onto the surface of granules creating areas of high concentration of organic particles (Bossier and Verstraete 1996). These areas of high concentration could be beneficial as they allow greater rates of substrate diffusion throughout the granule. Real wastewater presents a problem in its uncontrollable variability. Events within industrial, or even municipal, processes mean that the loading of compounds within a stream can vary significantly. This problem is further enhanced through biological deterioration; Schwarzenbeck *et al.* (2004) found that if the residence time within the wastewater storage vessel was too long only pre-digested feed was fed to the reactor. Granular systems however have been found to be able to cope with these variations in loading. While the ultimate aim of wastewater treatment research is to apply technologies to real wastes, due to the potential variability of real wastewater, and the difficulties this brings with developing real understanding of treatment fundamentals, synthetic wastewater was utilised within the scope of this study.

The effect of organic loading rate (OLR) in aerobic granular systems has been investigated (Moy *et al.*, 2002; Tay *et al.*, 2004a,b). Moy *et al.* (2002) used two reactors, one fed on acetate the other on glucose, with the OLR of these systems gradually increased throughout the study. For the acetate-fed reactor, granules began appearing 3 weeks after start up at 6 kgCOD/(m^3.d) and were compact and regular. However, as the OLR was increased to 9 kgCOD/(m^3.d) the granules became unstable and disintegrated. In the glucose-fed reactor, granules also formed after 3 weeks at 6 kgCOD/(m^3.d) but these had a fluffy outer appearance. As the OLR was increased these granules did not disintegrate, instead becoming smooth but irregularly shaped and growing from a mean diameter of 2.7 mm to 3.3 mm at 15 kgCOD/(m^3.d). The increase in OLR was also found to increase the settling velocity of granules and decrease the settled volume index (SVI), both of which are favourable characteristics for granules. Tay *et al.* (2004a) found that at low OLR of 1 and 2 kgCOD/(m^3.d) aerobic granules could not be formed. Granular sludge was generated at higher rates of 4 and 8 kgCOD/(m^3.d) but those under the higher OLR were unstable and were washed out of the reactor. Within the 4 kgCOD/(m^3.d) reactor a suspended solids concentration of 12 g/L was achieved with a SVI of 24 mL/g and a high removal rate of soluble COD of 99 %. Liu *et al.* (2003b) investigated the effect of OLR on the hydrophobicity of aerobic granules. Increasing the organic loading rate from 1.5 to 9 kgCOD/(m^3.d) found that there was little effect on the hydrophobicity of the granules. This shows that the OLR of the system needs to be maintained above a minimum threshold where aerobic granules have not been found to form, but below a maximum threshold where the granules may eventually disintegrate.

Calcium was found to have an affect on the ability of anaerobic systems to granulate. Precipitation reactions between calcium and phosphate were found to provide inert supports for bacterial growth. High concentrations of calcium may have adverse toxic effects. It was found that a calcium concentration up to 300 mg/L was beneficial however at concentrations greater than 600 mg/L calcium became detrimental to growth (Yu *et al.*, 2001).

2.3.2.4 Hydraulic Retention Time

The hydraulic retention time (HRT) of a system is an important characteristic of a granular system. Growth within a granular system can occur in the form of increased numbers of free-swimming bacteria, small flocs or an increased population on the surface of pre-existing granules. Shortening of the HRT results in higher levels of washout of the poor settling particles, such as the free-swimming bacteria and flocs, within the reactor (Morgenroth *et al.*, 1997; Beun *et al.*, 1999; Jang *et al.*, 2004; Pan *et al.*, 2004).

The volumetric exchange ratio (VER) of a system determines the proportion replaced during each cycle. The HRT of the system is reliant on both the VER and total cycle time following the following equation:

$$\text{Hydraulic Retention Time} = \text{Cycle Time} / \text{Volumetric Exchange Ratio}$$

Pan *et al.* (2004) evaluated the effect of HRT on the development of aerobically grown microbial granules. This study found that at low HRT (1 h) all the biomass within the system was washed out resulting in reactor failure. At a long HRT (24 h) the granule population was gradually substituted by bioflocs. Short HRT were also found to have decreased sludge production but the size of granules was found to be larger than at longer HRT. This shows that there is a definite balance to be achieved between both long and short retention times.

2.3.2.5 Aerobic Starvation

Aggregation and granulation have been found to be effective protection strategies for bacteria against starvation. Short periods under starvation conditions were found to increase the cell surface hydrophobicity of granules causing stronger cell-to-cell interactions and a denser, more stable structure (Bossier and Verstraete, 1996; Watanabe *et al.*, 2000).

Granules have the potential to carry out nutrient and carbon-removal in both oxygen rich and oxygen depleted environments. As the diffused oxygen levels within the granule decrease towards to the centre of the molecule, it is possible for anaerobic and anoxic zones to occur. Anoxic zones within aerobic granules have been found in which the removal nitrates through denitrification has been observed (Etterer and Wilderer, 2001; Beun *et al.*, 2001, 2002; Jang *et al.*, 2003; Yang *et al.*, 2003). The presence of oxygen impedes denitrification; however, the nitrifiers help keep the oxygen from penetrating into the centre of the granule so denitrification can occur (Beun *et al.*, 2001). With similar interference caused by oxygen to the nitrogen fixation process, it was hypothesised that the creation of low oxygen concentration zones within the granule would be ideal for the growth of nitrogen fixers.

The nature of SBR reactors means that the feed is pulsed into the system. This results in a feast/famine regime where directly following the feed stage the substrate concentration is high but then as this is consumed the microorganisms within the SBR are subjected to a period of starvation (Tay *et al.*, 2001a). This has been found to facilitate microbial adhesion and aggregation (Bossier and Verstraete, 1996; Watanabe *et al.*, 2000).

2.3.2.6 *Seed Sludge*

The inoculum for the majority of aerobic granular studies was sourced from municipal wastewater treatment plants (Morgenroth *et al.*, 1997; Etterer and Wilderer, 2001; Tay *et al.*, 2001a; Beun *et al.*, 2002; Dulekgurgen *et al.*, 2003; Jang *et al.*, 2003; Liu *et al.*, 2003b; Tsuneda *et al.*, 2003; Yang *et al.*, 2003, 2004; Qin *et al.*, 2004; Wang *et al.*, 2004). Sludge from standard SBR was also used as inoculum by Beun *et al.*, (1999) and Arrojo *et al.*, (2004).

Supplementation with specific bacteria has been found to improve treatment efficiencies within nitrogen fixing systems. Addition of nitrogen-fixing *Azotobacter* was found to significantly improve COD removal efficiencies in activated sludge. (Kargi and Özmihçi, 2002, 2004a & 2002b) and this could be implemented similarly with granular sludge. This would insure that the system would have an initial population of bacteria capable of fixing atmospheric nitrogen. This would greatly reduce the time required for a system to reach a stable nitrogen fixing population.

2.3.3 *Characteristics of Aerobic Granules*

2.3.3.1 *Morphology*

The diameter and surface structure of aerobic granules is largely determined by the substrate used as a carbon source, the organic loading rate that is being applied to the system and the hydrodynamic forces within the system.

In many cases, as aerobic granules grow larger they become more prone to shear stresses, either through bacterial decay, EPS consumption or production of organic acids and associated gases, and so are more likely to disintegrate (Morgenroth *et al.*, 1997; Wang *et al.*, 2004). Pores within the structure of the granule can cause it to become less dense, inhibiting settling and causing washouts (Morgenroth *et al.*, 1997). These pores may also become filled with biogas (such as those evolved through anaerobic processes) or other gases. These gases create a buoyancy force which can cause uplifting of the granule (Toh *et al.*, 2003).

EPS has been found to encapsulate granules, encouraging adhesion of cells to the granule, helping stabilise membrane structure and acting as a potential protective barrier (Liu *et al.*, 2004).

Toh *et al.* (2003) found that as aerobic granules increased in size, the settling velocity, and the overall density of the granule all increased (although not necessarily in linear proportion to the size increase). The increase in settling velocity can be attributed to Stoke's settling law (as follows) and it has been hypothesised that the density increase could be caused through accumulation of inorganic solids within a granule matrix. This equation applies as during the settling phase the reactors are subjected to quiescent conditions.

Stoke's law terminal settling velocity (Perry *et al.*, 1997):

$$u_t = \frac{g.d_s^2.(\rho_s - \rho)}{18\mu}$$

Where u_t = the terminal or free-settling velocity (m/s)

g = acceleration due to gravity (m/s^2)

d_s = diameter of the particle (m)

ρ_s = density of the particle (kg/m^3)

ρ = density of the medium (kg/m^3)

μ = viscosity of the medium (Pa.s)

Table 2.5 shows the sizes of granules found within aerobic granular systems. From this table it is evident that the size of granules has a high variability; the sizes vary from less than 0.5mm to over 6mm. The size appears to be dependent on the system characteristics, in particular the substrate and its loading rate as well as the settling time and turbulence.

Table 2.5 Granule Diameter values found within Literature

Reference	Mean Diameter (mm)
Beun *et al.* (1999)	2.10
Etterer and Wilderer (2001)	1.10 to 6.50
Tay *et al.* (2001)	1.10 to 2.40
Beun *et al.* (2002)	2.50
Moy *et al.* (2002)	1.96 to 4.20
Jang *et al.* (2003)	1.00 to 1.30
Tsuneda *et al.* (2003)	0.35
Yang *et al.* (2004a)	0.25 to 0.51
Yang *et al.* (2004b)	0.37 to 2.00
Su and Yu (2005)	1.22

2.3.3.2 *Settleability and Density*

The settling velocity of the granules and the SVI governs the settleability of granules. Faster settling granules with a low SVI have a better-defined solid-liquid interface allowing for more efficient solid-liquid phase separation. Table 2.6 shows sludge quality values from literature. The SVI values for granular systems are much lower than those reported for biofloc systems (as shown previously in Table 2.2 and Table 2.3).

Table 2.6 Granular Sludge Volume Index values found within Literature

Reference	SVI (ml/g)
Tay *et al.* (2001)	50 to 85
Moy *et al.* (2002)	31 to 106
Arrojo *et al.* (2004)	60
McSwain *et al.* (2004)	47 to 115
Schwarzenbeck *et al.* (2004)	30 to 40
Su and Yu (2005)	30.8
Tay *et al.* (2004)	20 to 60

Investigation into the settling velocities from granular studies (Table 2.7) found that there was significant variation in the rates the particles settled, which were caused by a large number of variables. Still, most studies found settling velocities between 0.8 and 1.0 cm/s.

Table 2.7 Granular Settling Velocities found within Literature

Reference	Settling Velocity (cm/s)
Morgenroth *et al.* (1997)	0.90 to 1.08
Beun *et al.* (1999)	0.67*
Etterer and Wilderer (2001)	0.35 to 2.00
Tay *et al.* (2001)	0.83 to 0.97
Moy *et al.* (2002)	1.47 to 3.11
Jang *et al.* (2003)	0.70 to 0.80
Zhu and Wilderer (2003)	0.83 to 1.94
Arrojo *et al.* (2004)	0.56
Wang *et al.* (2004)	0.91*
Su and Yu (2005)	1.02

*Denotes minimum required settling velocity within reactors

The typical range of specific gravities for aerobic granules has found to vary between 1.017 and 1.063 (Etterer and Wilderer, 2001; Tay *et al.*, 2004b; Su and Yu 2005).

2.3.3.3 Specific Oxygen Utilisation Rate

The activity of microorganisms is typically defined through the specific oxygen utilisation rate (sOUR). The sOUR can be used to compare activity rates between different systems.

When aerobic microorganisms respire, they consume oxygen, and the rate at which this consumption occurs is known as the specific oxygen utilisation rate (sOUR). The sOUR of different systems has been compared to determine the effect on the reactivity on biomass. Zhu and Wilderer (2003) used sOUR to determine the effect of extended storage time on the reactivity of granular activated sludge. Granules were stored for a 7 week period (under anaerobic conditions) before the system was restarted and after only a week of operation the system had returned to the same operating status as before the idle period.

2.3.4 Microbial Structure and Diversity

Advanced microscopic and analytical techniques have been used to determine the structure of granular surfaces and substructures of granules. The results showed that many different surface characteristics are possible. Depending on the system properties, in particular loading, substrate and hydrodynamic conditions, the surface was found to differ between several extremes. Some studies found granules to have smooth outer surfaces with hardly any filamentous growth (Moy *et al.*, 2002; Dulekgurgen *et al.*,2003; Jang *et al.*, 2003; McSwain *et al.*, 2004; Qin *et al.*, 2004; Yang *et al.*, 2004), other studies found granules to have star-like appearances (Morgenroth *et al.*, 1997; Tay *et al.*, 2001; Moy *et al.*, 2002) and some were found look like "balls of yarn" (Beun *et al.*, 1999; Schwarzenbeck *et al.*, 2004). Examples of these characteristic granules are shown in Figure 2.3.

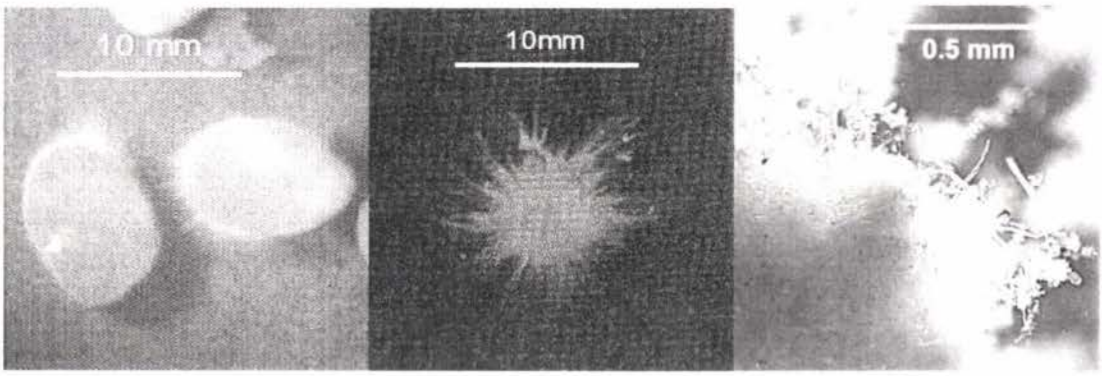


Figure 2.3 Granule pictures from literature. Left: smooth outer surface (Pan *et al.*, 2004); Center: large surface area, irregular surface (Morgenroth *et al.*, 1997); Right: "Ball of yarn" close structure highlights filamentous nature (Schwarzenbeck *et al.*, 2004).

Tay *et al.* (2002) used fluorescence *in situ* hybridisation (FISH) to investigate the spatial characteristics of granules with an average diameter of 2.4 mm. This study found that there were definite aerobic and anaerobic zones within a granule. At a depth of 70 – 100 μm from the edge of the granule an aerobic layer was situated. Channels and pores within the granule were detected throughout the granule with the majority occurring between 300 – 500 μm into the granule. Between 800 – 900 μm into the granule an anaerobic layer was detected.

2.3.5 Applications of Aerobic Granulation Technology

Aerobic granular systems have already been used to treat some real wastewaters. A number of studies have also been undertaken to determine its suitability for treating a diverse number of different types of wastewaters.

While some studies have shown that aerobic granules became unstable and disintegrated at high loading, Moy *et al.* (2002) demonstrated that they were capable of treating 92 % of a 15.0 kgCOD/(m3.d) loading rate for at least a two week period. This shows some of the variance inherent within granular systems.

It was found that simultaneous COD, nitrogen and phosphate removal by aerobic granules was also possible through selection of slow-growing organisms. Systems were stable over long periods (> 150 days) and also found that anaerobic feeding periods resulted in the formation of granules that were more stable (de Kreuk *et al.*, 2005).

2.4 Research Opportunity

Nowhere within literature has the treatment of nitrogen deficient wastewater using granular technology been investigated. This knowledge gap allows a great opportunity to develop a novel treatment process for these wastewaters and development of knowledge and understanding of interconnectivity between aspects in both technologies.

Nitrogen fixing technology has been found to be a promising solution for the treatment of nitrogen deficient wastewaters. It has, however, been found to have poor sludge quality. Biomass within nitrogen fixing systems has been found to be highly filamentous, which causes poor settleability of biomass.

The granulation of aerobic biomass has been investigated as a method of improving the settleability of a system while retaining its ability to treat wastewaters. Previous studies have found that a number of conditions both favour and preclude granulation of sludge occurring within a system. From these conditions a series of research questions have been generated.

2.4.1 What Effect does Settling Time have on the Selection, Size and Morphology of Biomass within an Aerobic System? (Chapter 5)

This chapter will detail the effect of settling time variation on the granulation process on aerobic systems run without nitrogen deficiency. The effect of settling time will be explored on a non-nitrogen deficient system in order to determine which settling time would likely give the most suitable results for a nitrogen deficient system.

2.4.2 What Effect does Extreme Nitrogen Deficiency have on Granulation within Carbonaceous Wastewaters? (Chapter 6)

This chapter will determine the effect of nitrogen deficiency on an aerobic granular biomass. Granulation of a nitrogen fixing system has, until now, not been researched. Investigation into the effect on size and morphology of granules and the overall treatment performance of the granular system will be carried out.

2.4.3 What is the Effect of Turbulence and High Oxygen Concentrations on Nitrogen Deficient Granular Systems? (Chapter 7)

This chapter will determine the effect that increased rates of turbulence have on nitrogen deficient granular systems. The nitrogenase enzyme that facilitates nitrogen fixation is inhibited by the presence of oxygen so a balance between the requirement for turbulence, for granulation to occur, and protection of the fixation mechanism may be required.

Chapter 3: Materials and Methods

3.1 Reactor Design

Granular reactors are usually designed to have a high height to diameter (H/D) ratio and this has been identified as a key parameter for the format of the reactor. Heating of the reactor was also required to attain temperatures suitable for growth.

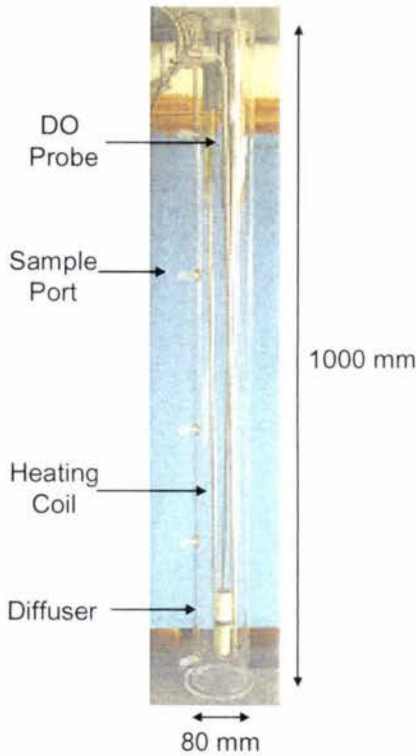


Figure 3.1 Reactor Design

The reactors were designed to be 1000 mm in height and 80 mm in diameter, representing a H/D ratio of 12.5. This is shown in Figure 3.1. For reactors A, B, C, and G the react volume was 4.3 L. For reactors D, E, and F 4.3 L was the fill volume with the bed height varying based on the amount of aeration (*refer to* Table 3.1).

Five ports were installed on the side of the reactor at 50, 250, 425, 650 and 850 mm from the base. These ports had an internal diameter set at 8 mm which was determined from literature as a size that should allow the granule to pass through unimpeded. Ports were clamped shut but allowed for access. From these ports both sampling and decanting was possible, with the port at 425 mm used for decantation at a volumetric exchange ratio (VER) of 50 %.

Heating and cooling, within the reactors, was done by passing hot and cold water through a stainless steel tube. The temperature within the reactors was controlled using a programmable logic controller (PLC) set at 30 °C.

Air was supplied into the reactors through a diffuser at the bottom of the reactors and controlled at a dissolved oxygen (DO) set point by a PLC.

Both the temperature and the DO were monitored using a sensor probe inserted from the top of the reactor.

3.2 Control System

Shown in Figure 3.2 is a screenshot of the operational PLC system for the granular reactors. Although the onscreen graphics were originally designed for a conventional SBR floc system, the information provided by the interface was sufficient for both control and monitoring of the systems. The important features of the screen are shown. Area 1 shows the status of pumps within the system. When the Button is set to "Auto" the pump will run according to the scheduled operation times – in this example the DO pump is off. Also shown is the weight of feed held within the chilled refrigeration unit. Area 2 shows the status of temperature control within the system. The positions of the hot and cold water inlets are represented and this shows whether and how much the valves are open. Area 3 reports the sensor information given by the DO probe as well as the setpoints for both the temperature and aeration. The percentage saturation within the system is reported as is the temperature (pH within the system was not measured). Area 4 details the current phase details: the length of time elapsed in the cycle as well as the actual and setpoint readings for temperature and oxygen saturation.

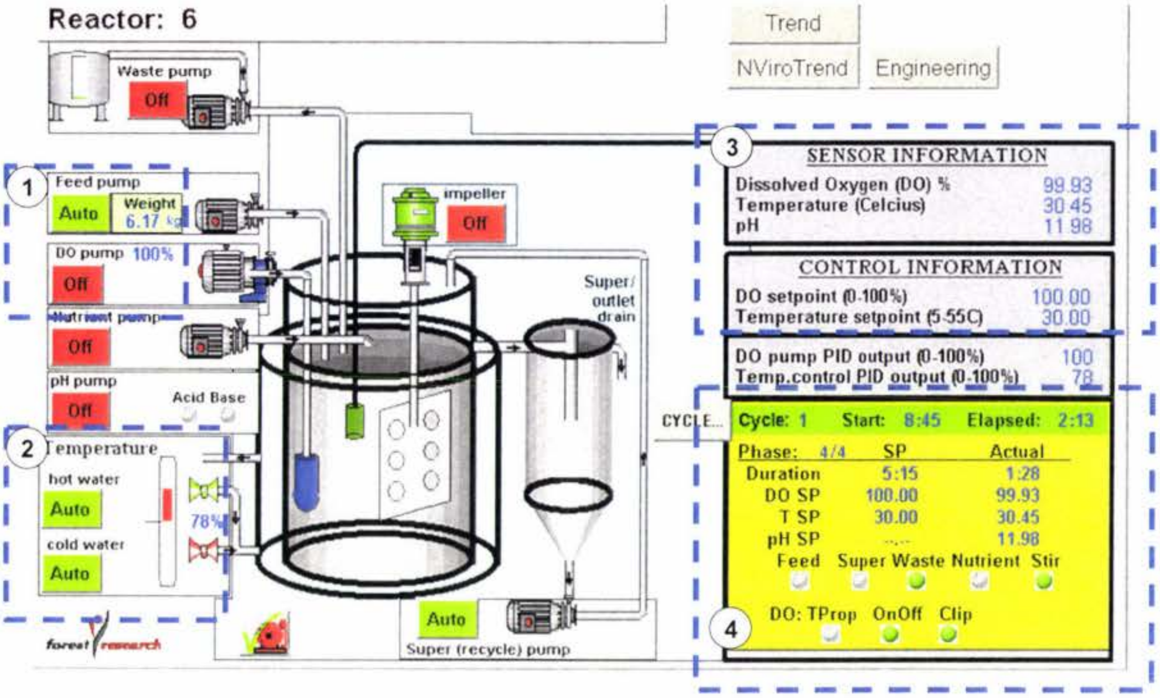


Figure 3.2 Control System Screen. Area 1: Feed and DO pump status; Area 2: Temperature control and valve activity; Area 3: Sensor Information; Area 4: Sequence information.

3.3 Reactor Operation

The reactors were run as sequencing batch reactors. Table 3.1 shows the experimental setup for the seven reactors run during the study and the conditions under which they were run. The high, moderate and low turbulence conditions were defined through upflow air velocities of 1.2 cm/s, 0.5 cm/s and less than 0.5 cm/s respectively (further detailed in *Chapter 7*).

Table 3.1 Reactor Conditions

Name	Settling Time	Nutrients	Turbulence
Reactor A	1 minute	N-Supplemented	Moderate
Reactor B	2 minutes	N-Supplemented	Moderate
Reactor C	15 minutes	N-Supplemented	Moderate
Reactor D	1 minute	N-Supplemented	High
Reactor E	1 minute	N-Deficient	High
Reactor F	1 minute	N-Deficient	Moderate
Reactor G	1 minute	N-Deficient	Low

The systems used in this study were run as sequencing batch reactors. The four phases within each cycle were:

1. Feed
2. React
3. Settle
4. Decant

While the Feed and Decant times were maintained at 30 and 15 minutes respectively, the settle time was varied between 1 and 15 minutes, and the react time was adjusted to provide a total cycle time of 6 hours. With a VER set to 50 %, this allowed the hydraulic retention time for all systems to be kept constant at 12 hours.

Temperature within the reactors was controlled throughout the cycle, while aeration was only provided during the react phase.

3.3.1 Feed Composition

Sodium acetate was identified as a desirable and readily available carbon source and was used as that basis for the synthetic wastewater used to feed all reactors. From previous studies and literature, the amounts of macro and micro nutrients required were calculated. It was discovered during the first set of experiments that no potassium was being added for a period due to a change in the phosphorous salt being added to the reactor, this omission was rectified by changing from Na_2HPO4 to KH_2PO_4. The original omission led to a deficiency in potassium and hindered some growth within the system.

The theoretical chemical oxygen demand (COD), based on the concentration of sodium acetate (2.055 g/L) within the feed, was set at 1600 mg/L. This corresponds to a organic loading rate of 3.2 kgCOD/(m^3.d). Within the nitrogen fed reactors the theoretical concentration of nitrogen was 57 mg/L.

Macro and micro elements were added as shown in the Table 3.2. Two important micronutrients within the feed were iron (II) and molybdenum. These two elements form coordination compounds that help protect the nitrogenase enzyme from exposure to oxygen.

Table 3.2 Final Feed Composition

	Compound	Concentration (mg/L)
Macro Nutrients	$CH_3COO^-Na^+$	2055.0
	$MgSO_4.7H_2O$	30.0
	$CaCl_2.2H_2O$	15.0
	$NaHCO_3$	6.9
	KH_2PO_4	35.1
	Nitrilotriacetic acid	15.0
	NH_4Cl (*for nitrogen fed*)	213.8
Micro Nutrients	$FeSO_4.7H_2O$	0.4005
	H_3BO_3	0.4650
	$CoSO_4.7H_2O$	0.1920
	$CuSO_4.5H_2O$	0.0165
	$MnCl_2.4H_2O$	0.0144
	$Na_2MoO_4.2H_2O$	0.1950

3.3.2 Inoculum

It was desired that the inoculum for the reactors, in particular the nitrogen deficient reactors, have a propensity for nitrogen fixation. From previous research (Clark *et al.*, 1997; Gapes *et al.*, 1999) bacterial communities from the treatment ponds of pulp and paper mills have the nitrogen fixing characteristics.

Reactors A, B, C and G were inoculated with seed microorganisms derived from three sources: a homogenised mixed liquor from a nitrogen deficient flocculating SBR; a solution of soil bacteria grown in an acetate-based feed mixture; and a mixture of liquor from two pulp and paper wastewater treatment ponds.

Reactors D, E, and F were seeded from non-granular biomass sourced from two SBR reactors, one of which was running under nitrogen deficient conditions and the other running under nitrogen sufficient conditions.

3.4 Mass Transfer Coefficient of Oxygen in Water ($K_L aO_2$) Determination

To measure the mass transfer coefficient of oxygen within the reactors they were filled with the feed mixture and the oxygen within the reactors was then purged by the bubbling of argon gas through the column. The dissolved oxygen concentration within the reactor was then measured as air was sparged into the reactor.

Using the following equation from Pratt *et al.* (2004) a value for the mass transfer coefficient was calculated.

$$\frac{dC}{dt} = K_L a_{O2}(C_{sat} - C)$$

Where: C is the dissolved oxygen concentration [mgL^{-1}]

t is time [hr]

$K_L a_{O2}$ is the volumetric mass transfer coefficient [hr^{-1}]

C_{sat} is the saturated dissolved oxygen concentration [mgL^{-1}]

Using the mass transfer coefficient it was then possible to determine the rate at which oxygen was being consumed during the biological reactions.

3.5 Sample Acquisition

Mixed liquor samples from the reactors were drawn from the ports located along the sides of reactors. From this primary sample sub-samples were taken for the various biochemical and image analyses.

3.6 Structural Characteristics

3.6.1 Image Analysis

Image analysis was carried out on samples to determine the morphological characteristics of granules within the reactor systems. Images were acquired through use of a digital camera and stereomicroscope and analysed through the ImageJ software package. The development of the image analysis processes is detailed within Chapter 4.

3.6.1.1 Digital Camera

Digital camera pictures used were acquired using a SONY DSC-F707 digital still camera with 10x Zoom and 5.0 Mega Pixel resolution.

Using the images acquired using the camera, the size and circularity distributions for each reactor were calculated.

3.6.1.2 Stereomicroscope

A Nikon CoolPix990 digital camera with 3x Zoom and 3.34 Mega Pixel resolution attached to a Leica MZ125 stereomicroscope was also used for microscopic observations of individual granules.

3.6.1.3 ImageJ

Pictures were processed using ImageJ, public domain image analysis software, and this process can be found in the following chapter. ImageJ itself can be downloaded from the http://rsb.info.nih.gov/ij/ website.

3.6.2 Population Characterisation

The Terminal Restriction Fragment Length Polymorphism (TRFLP) technique was investigated as a tool for determining the distribution of microbial populations based on the 16s RNA genes (Hiraishi *et al.*, 2000). TRFLP was primarily employed to identify differences in population within the nitrogen deficient and nitrogen supplemented reactors. Samples were frozen and sent to the EcoSmart Technologies Microbial Lab for analysis.

The procedure involves PCR amplification and fluorescent labelling of the environmental 16s RNA genes, digestion of the PCR product with restriction enzymes, and T-RF separation by automated electrophoresis.

3.6.2.1 Further Microscopy

Fluoresence in-situ hybridisation (FISH) was attempted. Samples were frozen and sent to the EcoSmart Technologies Microbial Lab for analysis. However due to the size and cellular density of specimens this technique was found to be inconclusive. Other aerobic granular studies have however made use of FISH with success (Jang *et al.*, 2003). This type of microscopy has the potential to develop understanding of the characteristics of nitrogen fixing aerobic granules and future work to investigate this should not be ruled out.

3.7 Biochemical and Treatment Characteristics

3.7.1 Suspended Solids

The suspended solids in the mixed liquor and supernatant were measured using a similar method to that outlined in APHA-AWWA-WPCF "Standard Methods". 25 mL of sample was filtered through pre-weighed GFC filter papers. The papers were then dried in an oven at 105 °C overnight before being reweighed and placed in a furnace at over 500 °C. This gives the values for total suspended solids and the volatile suspended solids for each of the samples

3.7.2 Carbohydrate

The carbohydrate concentration within samples was measured using a phenol-sulphuric colorimetric assay developed from Dubois et al. (1956). Phenol and concentrated sulphuric acid were added to the samples and reacted in a water bath at 30 °C for 10 minutes. Samples were then read using a spectrophotometer at 490 nm. Comparison of sample absorbance and glucose standard absorbance gives the equivalent carbohydrate concentration.

Three parts of each sample were analysed: the total mixed liquor, the filtered soluble fraction, and the third is the centrate sample. Using these values it was then possible to calculate both the EPS fraction (difference between the centrate and soluble values) and the solid fraction (difference between the total and soluble values).

3.7.3 Chemical Oxygen Demand

The chemical oxygen demand (COD) was measured using a similar method to that found in APHA-AWWA-WPCF "Standard Methods". The sample was digested using acidified potassium dichromate and measurement of the difference in absorbance following digestion gave the COD value.

3.7.4 Total Organic Carbon

The carbon concentration was measured using an Elementar High TOC II analyser. The analyser was capable of determining the total carbon concentration, the inorganic concentration and the suspended solids within the reactor. The difference between total and inorganic concentrations allowed the total organic carbon within the reactor was able to be calculated.

3.7.5 Nitrogen and Phosphorus

Total and soluble samples of the feed and effluent from the reactors were sent off for analysis of the phosphorus and nitrogen content. The analyses for phosphorus were for both total and dissolved; for nitrogen the analyses were for nitrates and nitrites, ammonia and dissolved and total Kjeldahl nitrogen. Samples were frozen and sent to the Veritec Analysis Lab for analysis.

3.7.6 Acetylene Reduction

The nitrogen fixing enzyme nitrogenase is capable of reducing acetylene to ethylene. A sealed container was flushed with argon to remove any residual air before having a small amount of acetylene injected in. A small amount of biomass was injected into the container and the sample left to react in a water bath. Head space from these containers was then injected into a gas chromatograph (1.5m, ¼" i.d. column containing Porapak N, 80/100 mesh)where the amount of acetylene and ethylene was measured.

3.7.7 Settling Velocity

Granules were placed in a quiescent cylinder (300 mm long and approximately 80 mm in diameter) filled with feed medium and the time take for the granule to settle 150 mm was measured.

3.7.8 Specific Gravity

Granules were immersed in a test tube filled with distilled water and a glucose solution (of known density and concentration) was titrated quickly into the test tube and the point at which buoyancy was achieved measured. From the titrated volume the density of medium could be calculated.

3.7.9 Sludge Volume Index (SVI)

Due to the nature of the reactors, deviation from standard methods was required. Mixed liquor contents were allowed to settle for only half the standard amount of time (15 min instead of 30 min).

The sludge volume index was determined by recording the volume of the sludge blanket after the 15 min settling period and then dividing the result by the total amount of solids within the reactor.

Chapter 4: Image Analysis Process

4.1 Introduction

In addition to the Literature Review on nitrogen fixation and aerobic granulation, an investigation into analytical techniques for characterising particles was carried out. This chapter effectively details the processes used to gain morphological data from the images taken of both individual granules and mixed liquor samples.

A number of studies have used image analysis to determine spatial characteristics of granules and other particles within wastewater systems (Li and Ganczarczyk, 1989; Zartarian *et al.*, 1997; Jeison and Chamy, 1998; da Motta *et al.*, 2001; Contreras *et al.*, 2004). The characteristics have included: area, perimeter, diameter, circularity (also known as the aspect ratio) and the fractal dimension (calculated using several different methods). These characteristics can help to classify and compare granules generated within different reactors and studies for similarities. This study used the image analysis software programme ImageJ (Rasband, 2006) as a method of acquiring and analysing pictures taken by both the digital camera and the stereomicroscope. The interface and availability of ImageJ were two key features why this programme was selected. Other studies have used similar analysis programmes; Jeison and Chamy (1998) analysed anaerobic granules with the UTHSCSA Image tool programme and Contreras *et al.* (2001) used Global Lab Image 2.10.

More specific studies have focused on the important edge characteristics and in particular the fractal dimension of a particle's edge. The fractal dimension has been used to describe the "ruggedness" of a particle or surface. As the line or edge of the particle becomes more tortuous the fractal dimension for the line increases. An increased fractal dimension can be associated with a greater surface area which is important for both reactivity and diffusion into the particle.

Included within this chapter is an overview of the statistical methods employed for analysis and the robustness of these results.

4.2 Sampling and Image Acquisition

Initially 10 mL of sample was withdrawn from sampling points along the side of the reactor, however due to the high concentration of particles and the overlap they caused this amount was reduced. A small, 2 to 3 mL, sample of mixed liquor was diluted down to cover the surface of an 80 mm diameter plastic Petri dish. Enough distilled water was added to fully cover any large particles within the sample. The Petri dish was then placed on an opaque backlight to prevent the creation of shadows. A mounted camera set 300 mm above the dish was used to take pictures of the Petri dish and a 10 mm micrometer scale bar placed next to the dish. On each sampling date, pictures of two samples from each reactor were taken, and the resulting data compiled and analysed.

Another set of pictures was taken using a digital camera attached to a microscope. These images were of a significantly higher magnification but were only able to capture a small number of particles within each picture.

4.3 ImageJ

The pictures taken were edited using ImageJ using the following steps (these steps are also shown in Figure 4.1):

1. Conversion of the image from colour to greyscale.
2. Scale is set using the micrometer scale bar.
3. Subtraction of background to remove the inherent "noise" of the image.
4. Cropping of image to remove areas of image outside of the Petri dish.
5. Conversion of image to binary black and white using the threshold function.
6. "Analyse Particles" function run, a minimum particle size of 40 pixels was set, this corresponds to the 200 μm size that was found to be a detection

limit for the camera. This analysis function returns data on the area, circularity, perimeter and Feret's diameter of the particles.

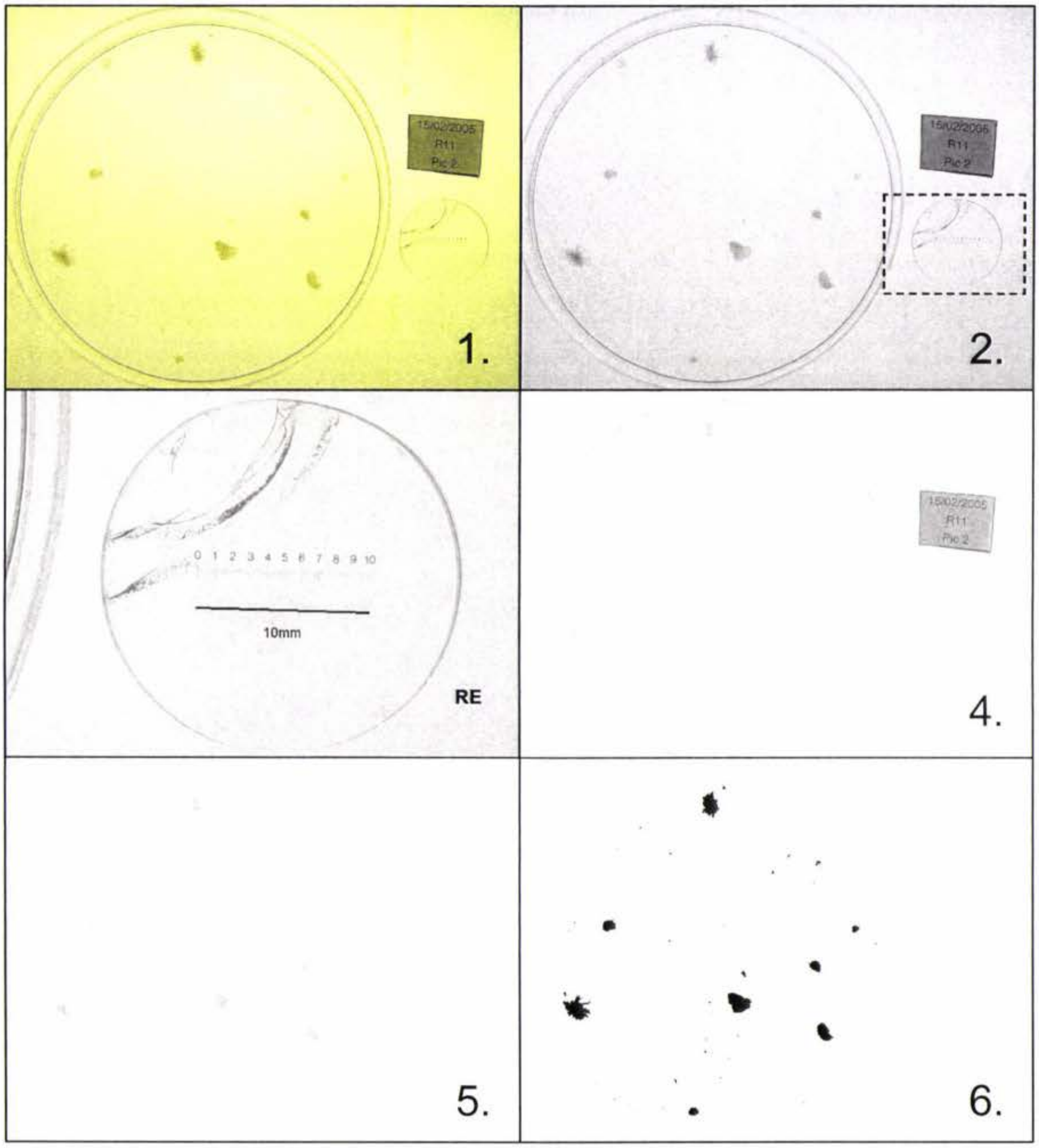


Figure 4.1 Step by step example of the image analysis process: 1. Initial image; 2. Gray scale; 3. Scale acquisition; 4. Subtraction of background; 5. Cropped image; 6. Conversion to black and white.

The Analysis Particle function in the software was set to determine the following parameters:

- Area: calculated based on the number of square pixels within an object.
- Perimeter: the length of the outside boundary of an object.
- Feret's Diameter: this is defined as the longest possible distance between any two points on the object's perimeter.
- Circularity: this is defined as Circularity = 4π*(Area/Perimeter^2), for a circle this identity has a value of 1, as the circularity of an object approaches 0 it becomes increasingly elongated.

The area, perimeter and Feret's diameter, were recognised as primary characteristics, while the circularity is a derived secondary characteristic. These characteristics are important as they allow comparison of the effect changes in settling time, turbulence, and nitrogen concentration, have on the particles within the reactors.

4.4 Statistical Size Comparison of Samples

Sample data from Reactors A, B and C were used to investigate and demonstrate the analyses carried out to determine granule size and structure.

The size of granular particles is important. Stoke's Law states that as the granule's radius increases the settling velocity also increases. Increased settling velocity allows more of these larger particles to remain within the reactor despite short settling times. Larger particles also allow for a greater range of microorganisms to grow, as oxygen and chemical concentration gradients within the granule differ.

Initially two samples were taken for each reactor and from each sample two pictures were taken after mixing. Using the previously mentioned ImageJ methodology the characteristics of the granules growing within the reactors were measured. The equivalent diameter of each particle was derived assuming a circular relationship through the following formula:

$$\text{Area} = \frac{\pi.\text{Diameter}^2}{4}$$

Figure 4.2 shows the mean and median values for each reactor, each sample and each picture. The difference between the two values shows the skewness of the particle size distribution.

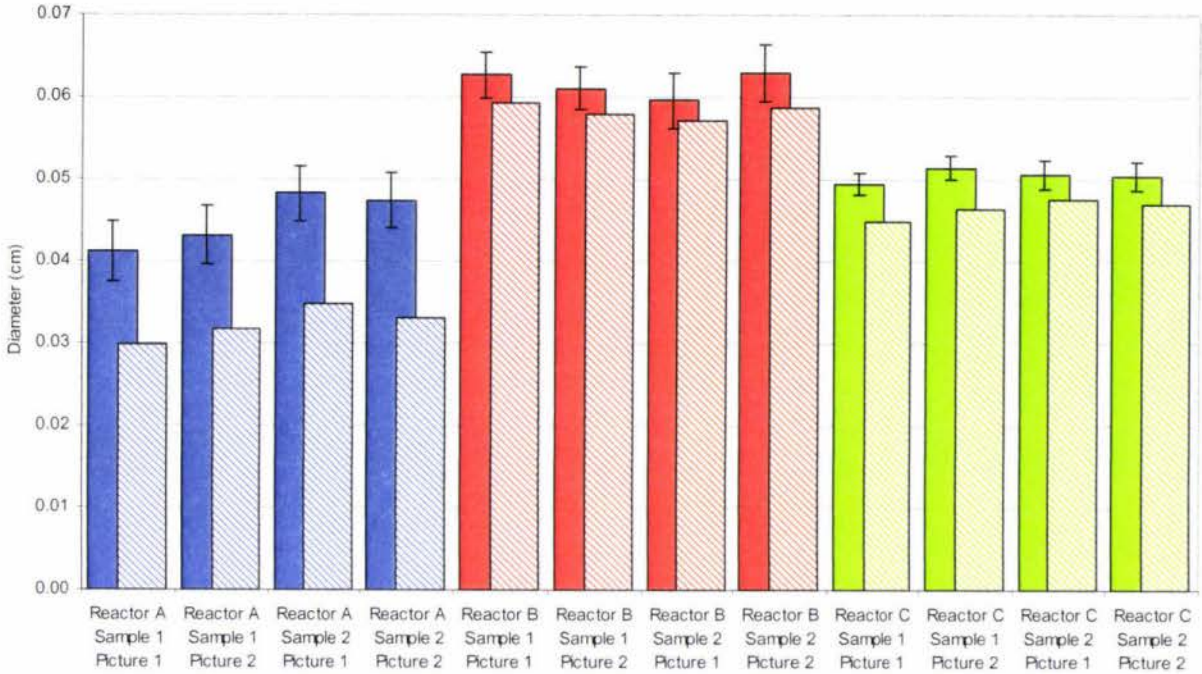


Figure 4.2 Comparison of the equivalent diameters of particles.
Key: ☐ Mean Values with 95 % Confidence Intervals
▨ Median Values

The information shown in Figure 4.2 also shows that, for Reactors B and C, quoting either the mean or median value gives a similar value for the sample population. However, for Reactor A, this is not the case with a much greater variation between the mean and median value. This shows that there must be some care given to the way in which these values are reported.

As shown by the mean value error bars the difference between both the pictures of the same sample was insignificant. The difference between pictures was also found to be low, the difference between pictures of Reactor A are more noticeable but the confidence intervals still overlap.

The large number of particles within the pictures was found to lead to 'clumping' of particles together. This agglomeration of particles led to an increase in the mean size and decrease in the overall particle count. To avoid this problem, the amount of sample analysed was reduced and this resulted in the differences between pictures that were caused by clumping to be greatly reduced.

Figure 4.3 below shows the distribution of granules based on their mean radius dimension. The peak distribution of the granules occurs at or below 0.2 mm for each reactor. Due to this large number of smaller particles, the median radius of the system is lower than the mean value (as shown previously in Figure 4.2). In systems such as these the minority of larger granules has a greater influence on the mean value than on the median value.

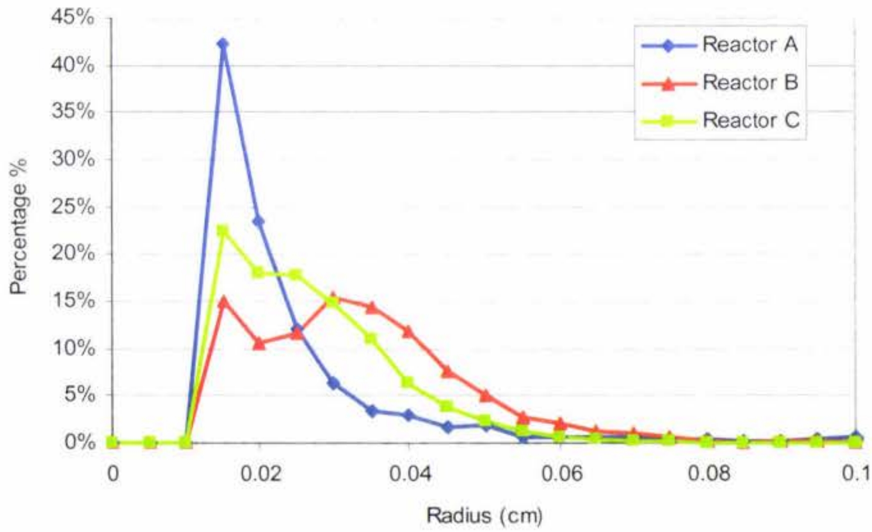


Figure 4.3 Population distribution based on the radius of granules

Figure 4.4 shows the cumulative particle size distribution weighted by the equivalent volume of the particle (calculated from the particle area). This graph weights larger particles much higher than smaller particles and gives a much different idea of the distribution of biomass within the reactor. The weighting employed by this graph is important as effects within the system are influenced more by the larger particles than smaller particles as the amount of biomass within larger granules is exponentially greater than smaller granules.

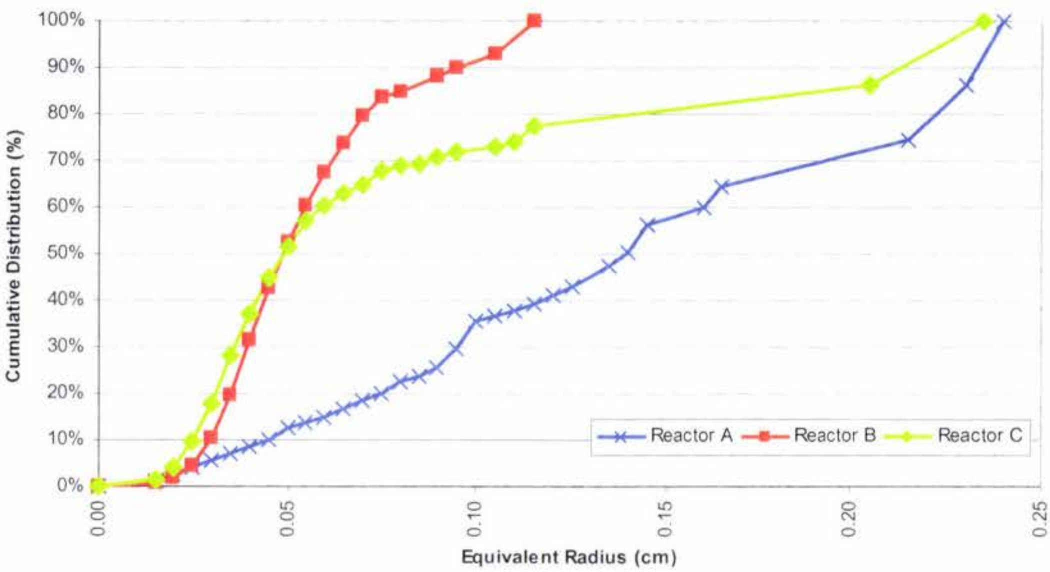


Figure 4.4 Cumulative Distribution graph on a volumetric basis.

4.5 Circularity

The turbulence and shear forces within the reactor have been found to exert dramatic influence over the formation of granules. The particle circularity was found to show the influence that turbulence and other driving forces have on the morphology of granules.

Shown in Figure 4.5 are the radius and circularity of particles within each reactor. No relationship was found between the circularity and the size of particles.

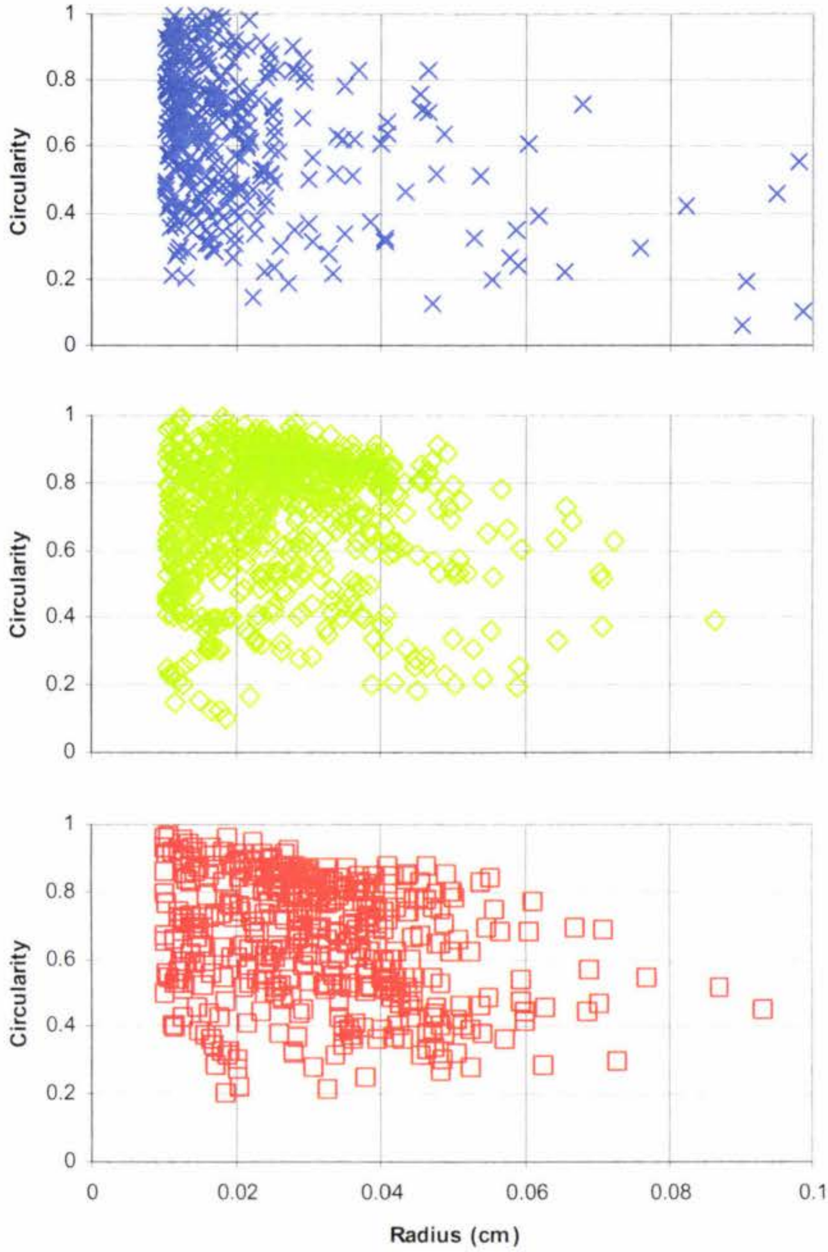


Figure 4.5: Circularity and Size Correlation. Top: Reactor A, Middle: Reactor B, Bottom: Reactor C.

Table 4.1 shows the statistics obtained from the image analysis into the circularity and Figure 4.6 a graph representing the mean circularity of the reactor. This data shows the similarities between Reactor A and Reactor B, with mean circularities of 0.631 and 0.658 respectively, and the increased circularity of particles within Reactor C, with a mean circularity of 0.697.

	Reactor A	Reactor B	Reactor C
Mean	0.6313	0.6584	0.6973
Standard Error	0.0070	0.0068	0.0039
Median	0.6523	0.6852	0.7546
Mode	0.8803	0.7954	0.8948
Standard Deviation	0.2251	0.1852	0.1930
Sample Variance	0.0507	0.0343	0.0372
Kurtosis	-0.7042	-0.6655	-0.2733
Skewness	-0.3840	-0.4988	-0.7856
Range	0.9374	0.8370	0.9022
Minimum	0.0622	0.1572	0.0942
Maximum	0.9996	0.9942	0.9964
Sum	651.49	485.25	1674.96
Count	1032	737	2402
Confidence Level(95.0%)	0.0137	0.0134	0.0077

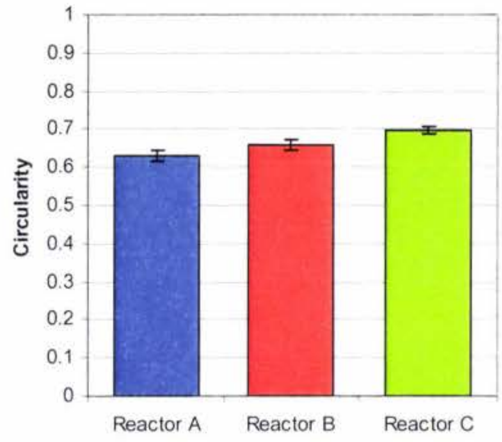


Table 4.1 and Figure 4.6 Descriptive statistics of the circularity of particles and graph of the mean circularity with 95% error bars.

From the descriptive statistics, generated using Microsoft Excel, the kurtosis value was used to identify how the population within the reactor was distributed. Larger, more positive kurtosis values, known as leptokurtic, show that the population distribution is 'peaked' around the mean value, but with an increased probability for extreme values. Negative kurtosis values, platykurtic, represent well rounded peaks with smaller tails and lower probability of extreme values. A zero kurtosis value, mesokurtic, has been found to represent normalised population distributions.

From Table 4.1 it is shown that all three systems are not normally distributed but the kurtosis values of -0.70, -0.67 and -0.27, for Reactors A, B and C respectively, describe the populations as platykurtic in distribution. These distributions are shown in Figure 4.7. The kurtosis values are shown to offer accurate descriptions of the population distribution in that for each of the systems there are no well defined peaks; Reactor C, which does show a slightly defined peak has the highest kurtosis value.

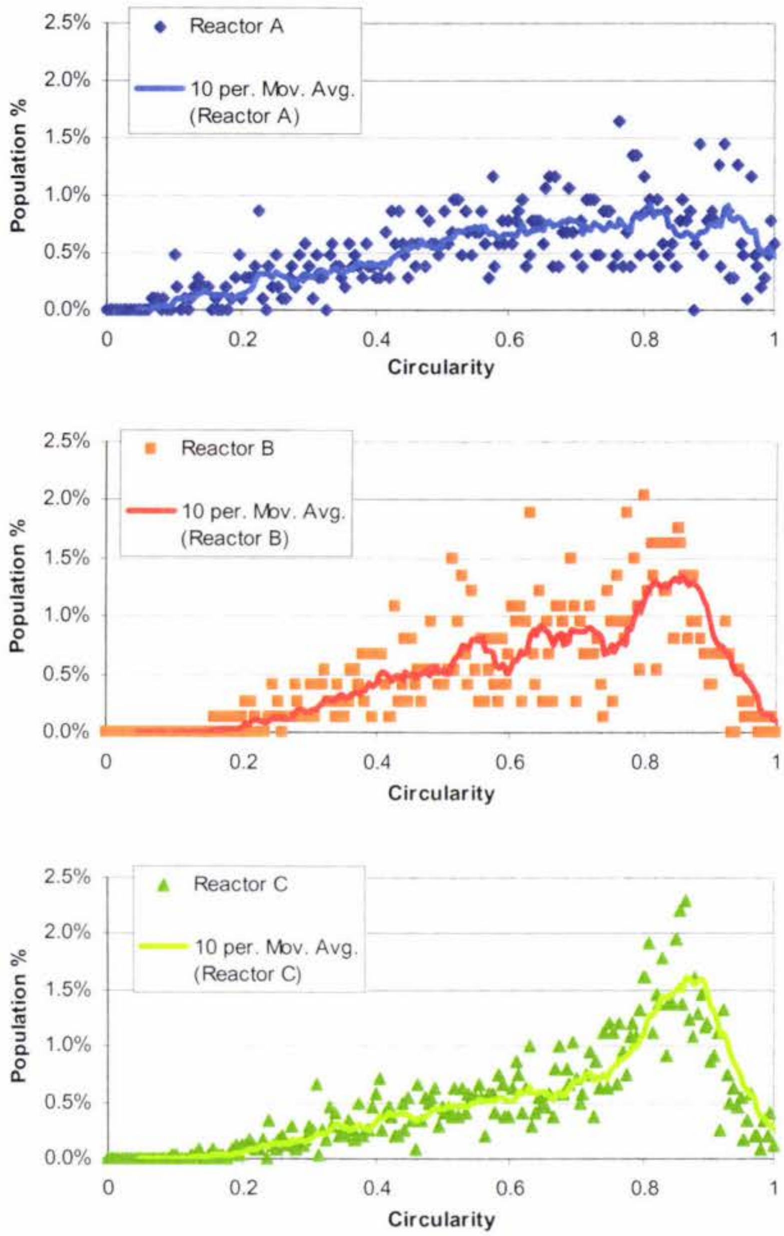


Figure 4.7 Population distribution based on the circularity of particles

4.6 Fractal Dimension

The typical way to describe and define fractal dimensioning is through the use of an island analogy. From a distance it is possible to describe the coastline of the island as a series of joined "smooth" curves. However, as the magnification on the edges of the island is increased it becomes more evident that each curve is not smooth but the 'average' of the irregularities makes it appear so. Zooming in further it is possible to tell that these irregularities are themselves made up of plants, rocks, and even buildings. Zooming further in to look at smaller and smaller parts of the image the complete measurement of the coastline continues to increase as the number of irregularities increase. The fractal dimension refers to this increase in perimeter as the magnification increases (Kaye, 1994).

Shown in Figure 4.8 are lines of different fractal dimension. Clearly as the fractal dimension of the line increases the tortuosity increases as well.

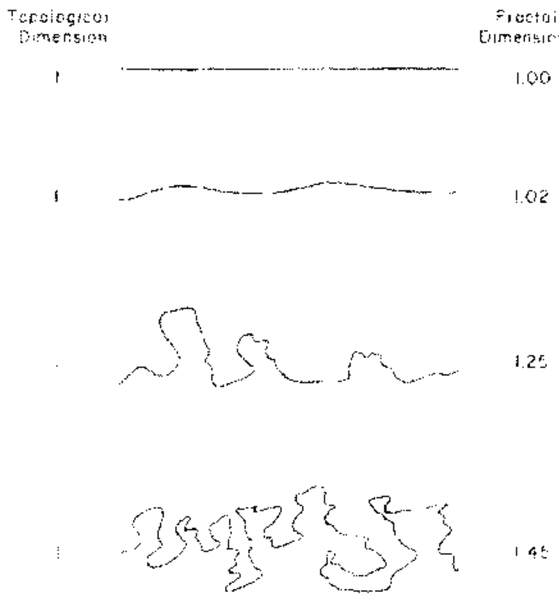


Figure 4.8 The relationship between line ruggedness and fractal dimension (Kaye, 1994).

The literature review revealed that granular particles can have different edge characteristics. Those subjected to high shear conditions were found to be smoother, and more spherical than those subjected to less turbulence.

To determine the fractal dimension of particles Zartarian *et al.*, (1997) used an area-perimeter relationship:

$$P \quad k.A^{(Df/2)}$$

Where: A Area

P Perimeter

Df Fractal coefficient

k Proportionality constant

Using this relationship, graphs of log A vs. log P were constructed to determine the fractal dimension coefficients. From these slopes the following coefficients shown in Table 4.2 were found. For a standard (non-fractal object) the Df identity should have a value of 1. Comparison of the three reactors shows that Reactor A is significantly more fractal than the other two reactors.

Table 4.2 Fractal Dimension Characteristics

Reactor	Df	k
A	1.252	17.87
B	1.081	12.06
C	1.033	10.80

This is a fair description of what was seen in the reactors: Figure 4.9 shows that the granules from Reactor A have a much more irregular edge characteristic.

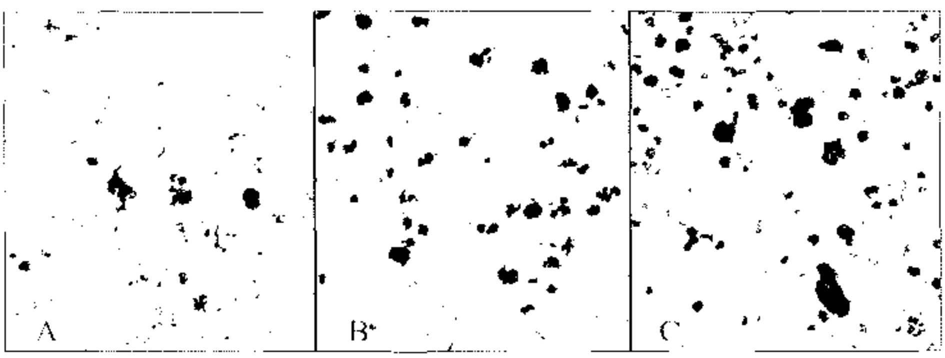


Figure 4.9 Sample analysed for the fractal dimension from Reactors A (left) B (middle) and C (right).

The Zartarian *et al.* (1997) relationship reveals the fractal dimension of a population of entities, and cannot be applied to the individual granules. For the analysis of the fractal dimension of individual granules a different correlation (coded as the FracLac plug-in for ImageJ) was used. Figure 4.10 Shows two granules with different fractal dimensions calculated using the FracLac plug-in. The fractal dimension for these granules was 1.5665 and 1.6552 for granule a) and b) respectively. Again, from these images it is clear that the granule with the higher fractal dimension has a distinctly more irregular and rough edge.

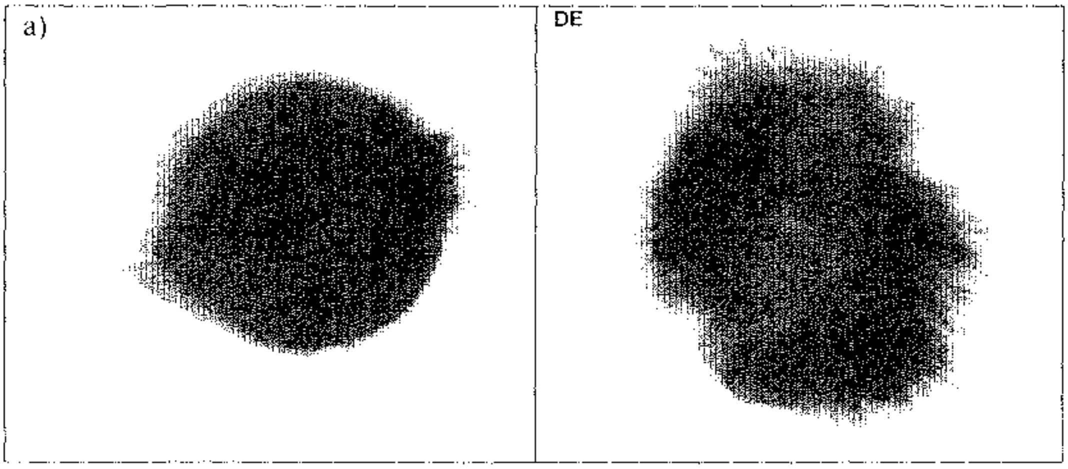


Figure 4.10 Examples of two granules with different fractal numbers. granule a) has a fractal dimension of 1.5665 and granule b) of 1.6552.

4.7 Summary

In an effort to help classify granules image analysis was used to describe the physical characteristics of granules. Using the freeware image analysis software ImageJ, it was possible to quantify characteristics for individual granules as well as for multiple granules within a sample. Characteristics such as granule radius, circularity and fractal dimension were quantifiable.

Samples were gathered using a standard method and images taken using a digital camera.

Investigation into the statistical differences between samples found that the variance between samples was less than the variance within the sample itself. Similarly an investigation into the variance created between pictures of the same sample was also found to be insubstantial.

The biomass within the system was held predominantly within the larger granules. Using a volume-based frequency graph for the granular population was assessed as a better method for determining the 'average' granule size.
The kurtosis values can be used to describe the type of distribution of a population around a mean. This allows a quantitative and comparative assessment of how the granules are distributed within the different systems.

The circularity of the particles was also investigated. No relationship was found to exist between the size and circularity of particles. So a standard population frequency graph of the circularity was able to be used to find the peak values for each reactor.

Using a perimeter-area relationship the fractal dimension for the same picture sample used for the size distribution and circularity values was able to be calculated. Further investigation into the fractal dimension values for individual granules was also possible using stereomicroscope images. The fractal dimension itself was able to describe the edge characteristics of the granules analysed.

Chapter 5: Effect of Settling Time on Granulation

5.1 Introduction

Sequencing batch reactor systems rely on a 'settle' phase for effective solid-liquid separation. The period of time required for separation is a function of the settleability of the biomass and thus directly influences the settling time within the system. Importantly, it directly effects the total cycle time; a reduced settling time would enable increased throughout. Equally significant, the settling time itself may influence the settleability of biomass within the system. Several studies (Beun *et al.*, 2002; Wang *et al.*, 2004) have noted that a reduction in the settling time has the ability to increase the preferential selection of granules. Further studies (McSwain *et al.*, 2004; Qin *et al.*, 2004) found that if the settling time was not short enough the suspended sludge within the system was not washed out, and granules were unable to become dominant within the system.

A suitable reduction in the settling time could result in significant increases in the amount of wastewater passing through a system per unit of time. It is suspected that this reduction would also lead to an improvement in the sludge settleability (with the formation of granules) and so would result in an increase in system efficiency without compromising the treatment given to the wastewater. In this chapter, the effect of settling time on biomass granulation is reported. The main aim of this thesis was: to produce granulation on nitrogen deficient feeds, and the work (nitrogen supplemented feeds) discussed in this chapter acted as preparatory work for setting appropriate conditions for that study (described in Chapter 6)

5.2 The Reactors

To determine the effect of settling time on the production of granules within the SBR systems three aerobic nitrogen supplemented reactors were run under settling times of 1, 2 and 15 minutes (Reactors A, B and C respectively). As these reactors were the first attempt at attaining granulation, no attempt to also achieve nitrogen fixation was made. The reactors were run for over 60 days which corresponds to 120 hydraulic retention times (HRT). To keep the total cycle time of each reactor constant, the react time within each cycle was adjusted to maintain 4 cycles each day (6 h total cycle time). This was done so that the HRT within each reactor would be identical as this has been shown to have some effect on the formation of granules.

As the reactors are "well-mixed" there is a theoretical possibility for particles to be present at any point within the reactor. This means that any particle has a chance to be retained in spite of bad settling properties if its position within the reactor is below the decant line. For a particle to settle from the very top of the reactor (Figure 5.1) to below the decant line a minimum settling velocity, as shown in Table 5.1 for each reactor, was required.

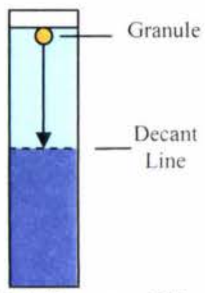


Figure 5.1 Reactor Diagram: For a particle to remain within the Reactor it must settle below the Decant line in the allocated settling time or face wash out

Table 5.1 The Settling Times and the Velocities required for a Particle to Settle from the Top of the Reactor

Reactor	Settling Time (min)	Required Settling Velocity (mm/s)
A	1	7.1
B	2	3.5
C	15	0.5

This theoretical comparison shows that the required settling velocity within the short settling time reactor (A) is much higher than in Reactor B, which is again much higher than in Reactor C. Literature shows granules have been generated in systems where the required settling velocity has been as low as 6.7 mm/s (Beun *et al.*, 1999). This means that the effect that washout has on shorter settling time reactors should be much greater than in reactors with longer settling times. So, it was expected that all three reactors would demonstrate significantly different granule and biomass populations due to the difference in settling time. However, specific treatment performances (g carbon removal per g biomass) were expected to be similar within all three reactors.

Initially the reactors were only fed a fraction of the final carbon (acetate) load. This fraction was gradually increased so that on Day 11 the full carbon load was being added. During the initial period, potassium was inadvertently not added to the feed. Growth within the reactors occurred despite this deficiency. This was attributed to long retention of biomass within the system and endogenous recycling of any potassium inherent within the system. Potassium was added to the system for the final 3 weeks of operation. The system evolution and steady state acclimation are split at the point where this potassium was added.

5.3 Evolution of the System

As biological systems are run, they continue to mature and evolve until the reactor reaches a steady state condition, where changes within the system become entirely responsive to the changes made on the system.

5.3.1 Initial Conditions

All three reactors started from the same inoculum mixture (see Chapter 3). Almost immediately the effect of differing the settling time on the population within the reactors became evident. Shown in Figure 5.2 is a sample of the biomass within each of the reactors on Day 2. These pictures show that the amount of biomass remaining within each reactor is directly related to the amount of washout caused by the settling time. Reactors A and B, with their respective 1 and 2 minute settling times, have much less biomass than Reactor C with its 15 minute settling time. The washout of particles, especially in Reactors A and B, allows for the continual selection of faster settling particles.

The particles remaining continue to grow during the react phases increasing in size and mass, with these increases the particles are more likely to remain within the reactor.

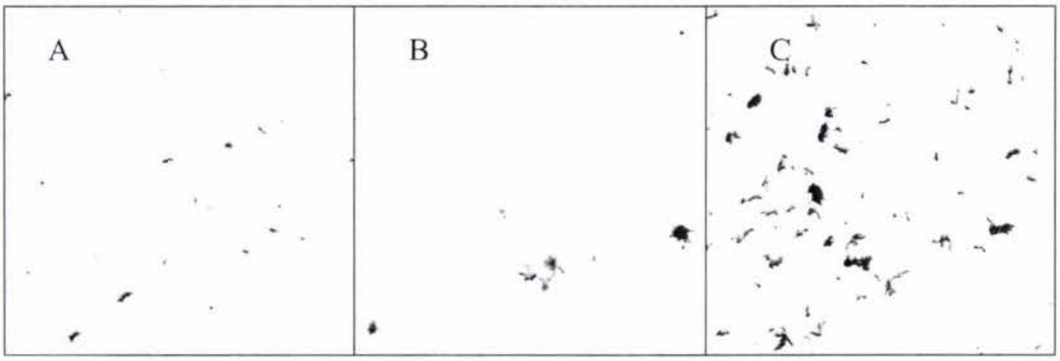


Figure 5.2 Day 2 Reactor samples from Reactor A (left, 1 minute settling time) Reactor B (centre, 2 minutes) and Reactor C (right, 15 minutes).

5.3.2 Morphological Characteristics

The evolutionary progression of the biomass within each reactor was documented through changes in images taken during development.

Pictures of the biomass within each reactor were used to document the evolutionary progression of the biomass and granules which formed. Figure 5.3 shows a selection of the biomass images taken from the initial stages through to their final form.

The pictures from Day 11 show that already the settling time had a significant impact on the development of biomass characteristics within each reactor. While the population within Reactor A (1 minute settling time) and Reactor B (2 minute) had developed into large particles, the biomass within Reactor C (15 minute settling time) is significantly smaller and there are significantly more individual particles.

The images taken on Day 27 highlighted the similarities between the biomass within the 2 minute and the 15 minute settling time reactors. While granules within Reactor A continued to increase in size, and lost some regularity of shape, particles within Reactor B decreased to a comparable size as those within Reactor C. Although the number of these small particles was still higher in Reactor C, there was a decrease from the amount found on Day 11.

During the period between Day 27 and Day 48 the addition of potassium was made. Following this, a significant change occurred within all three reactors. The number of granules within Reactor A decreased, but the biomass concentration within the reactor remained similar (as *shown later in Figure 5.11*); granular particles within both Reactor B and C increased perceptively in size although some flocculent biomass was present (images were unable to successfully convey this biomass fraction). While this change in population can be attributed to maturation of the reactor the introduction of potassium was likely to also have had an effect.

The sizes of granules within Reactor A remained stable, with slight variation, for the continuation of the study. By Day 63 some larger granular bodies were present within Reactor B, but still there was a significant amount of smaller granules and flocculent biomass present. Reactor C had a very similar biomass population to that found in Reactor B although the increased retention of biomass resulted in an increase in the amount of flocs retained within the system.

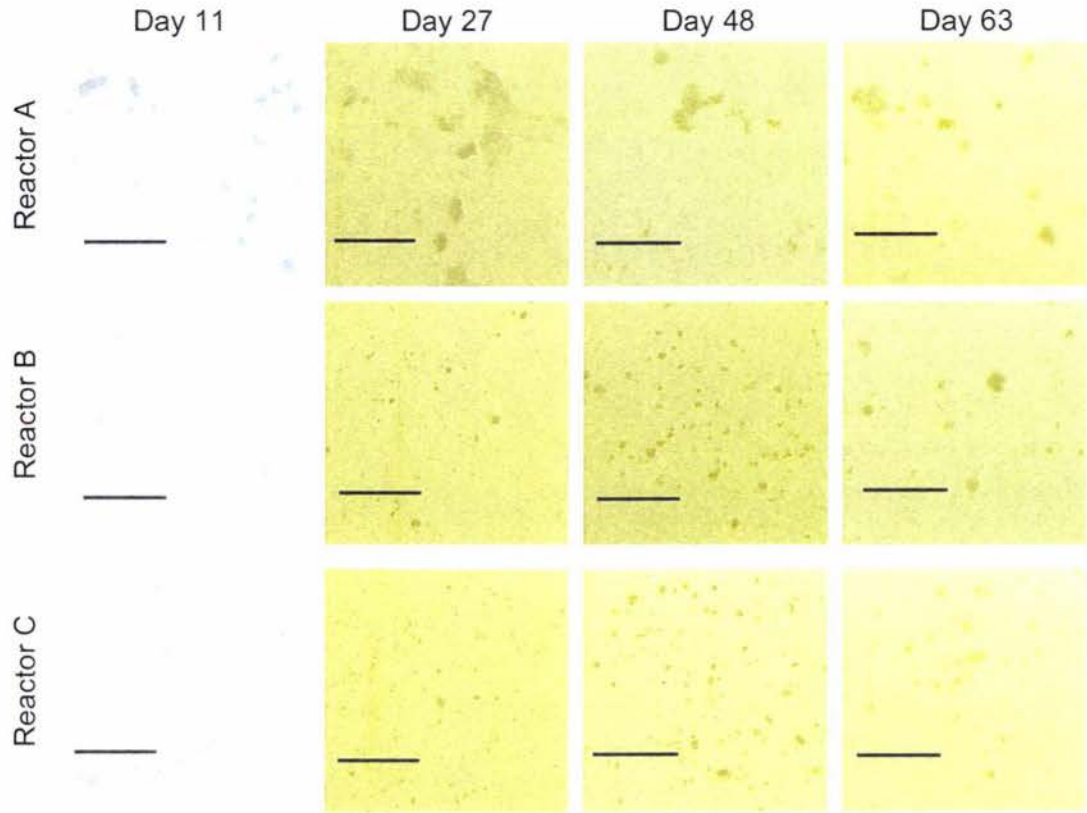
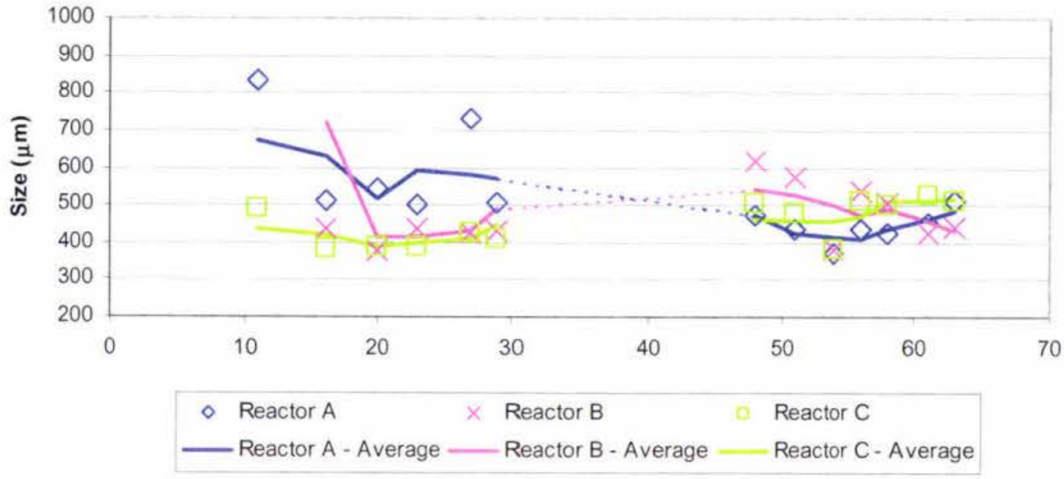


Figure 5.3 Granule Images. Top row shows the evolution occurring within the 1 minute settling time reactor (A), the middle shows the 2 minute settling time reactor (B), and the bottom row represents the reactor with a 15 minute settling time (C). The bar represents 10 mm.

5.3.3 Particle Size Distributions

Shown in Figure 5.4 is a graph of the evolution of granule mean diameter (on a basis where each particle has equal weighting) and their kurtosis based distributions. Despite some initial fluctuation in the mean size of granules in all three reactors the end mean diameter of particles within all three reactors was found to vary between 400 and 500 μm. Of more interest are the kurtosis values which show how the population distributions vary over time. Granules in Reactor A become increasingly similar with fewer extreme particles. In both Reactor B and Reactor C the distribution is more normalised. This shows that settling time selection is not significantly affecting the population within the Reactors B and C but does have an impact on Reactor A.



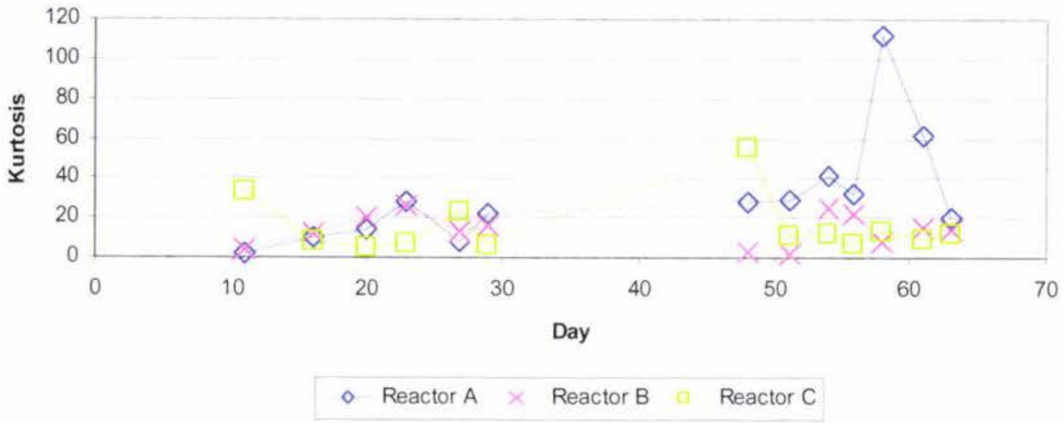


Figure 5.4 Mean diameter and kurtosis values for Reactor A (1 minute settling time), Reactor B (2 minute settling time) and Reactor C (15 minute settling time).

5.3.4 Particle Circularity Distributions

Investigation into the circularity of granules emphasised again the difference between Reactor A, with 1 minute settling time, and Reactors B and C, with 2 and 15 minute settling times respectively (Figure 5.5). All three reactors started from similar circularities and then after three weeks of operation the divergence of biomass occurred. Biomass within Reactor A was found to be significantly less circular (with a final mean circularity of 0.62) than the biomass within the other two reactors (final mean circularities of 0.69 and 0.70 for Reactor B and Reactor C respectively) and these distributions are shown in detail in Figure 5.6. The kurtosis values show that the distributions around these means are almost normalised: mesokurtic distribution with a trend towards a platykurtic spread, especially shown by Reactor A.

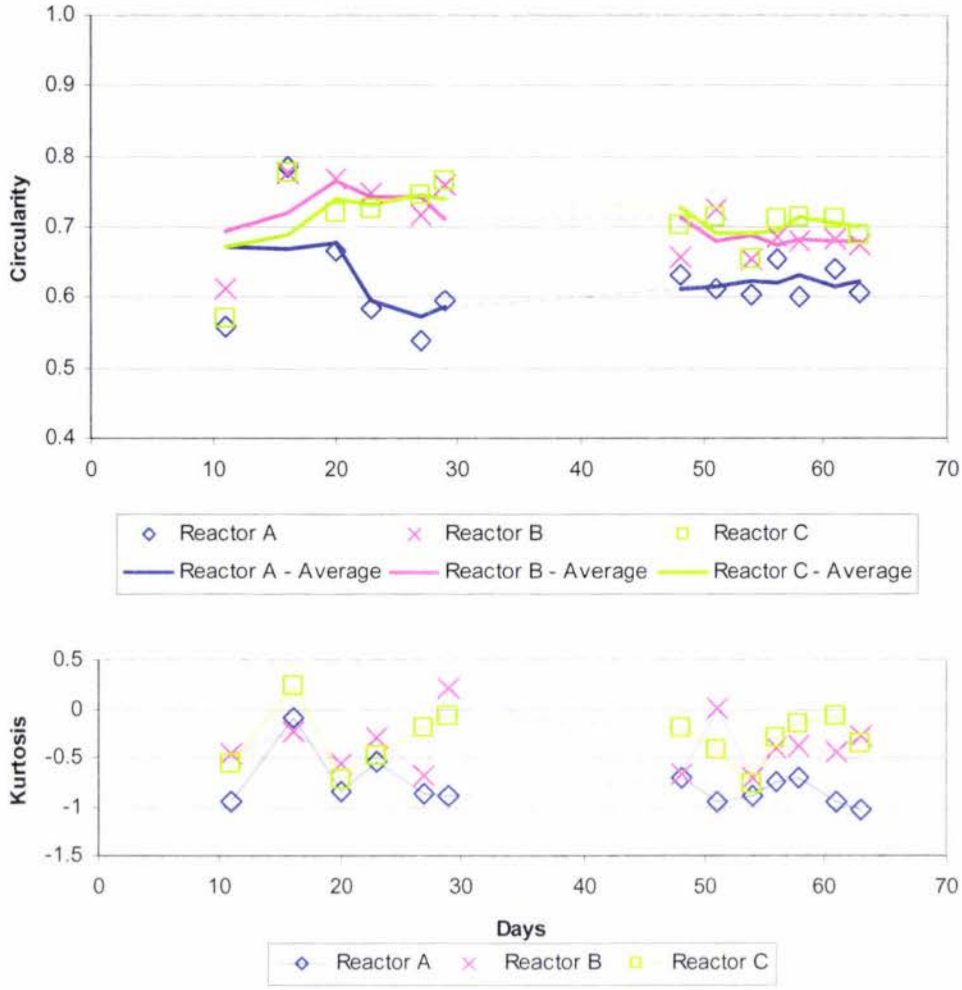


Figure 5.5 Mean Circularity and kurtosis values for Reactor A (1 minute settling time), Reactor B (2 minute settling time) and Reactor C (15 minute settling time.

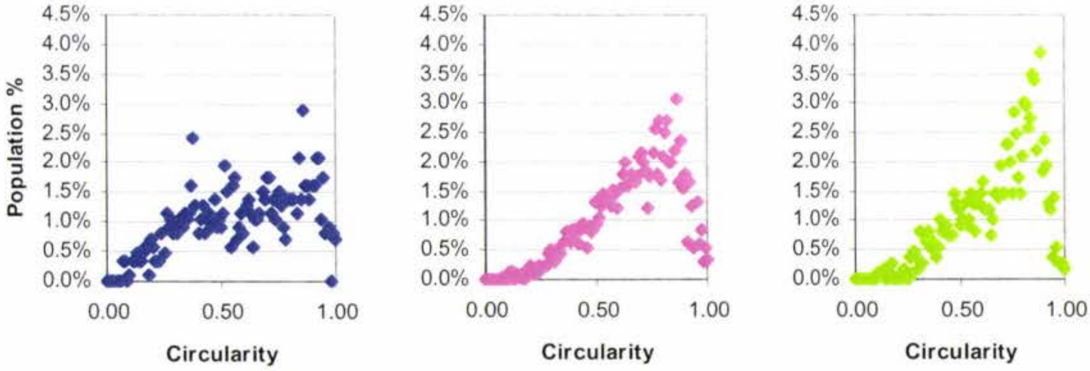


Figure 5.6 Day 63 circularity distributions for Reactor A (left) Reactor B (middle) and Reactor C (right).

5.3.5 Particle Fractal Characteristic

The fractal dimension can be used to quantify the 'ruggedness' of granules. This is important as the more irregular the edge characteristic of a particle is, the greater the surface area of that particle. This has been noted to enhance the treatment characteristics of granules with wastewater systems as there is a greater active portion of a particle.

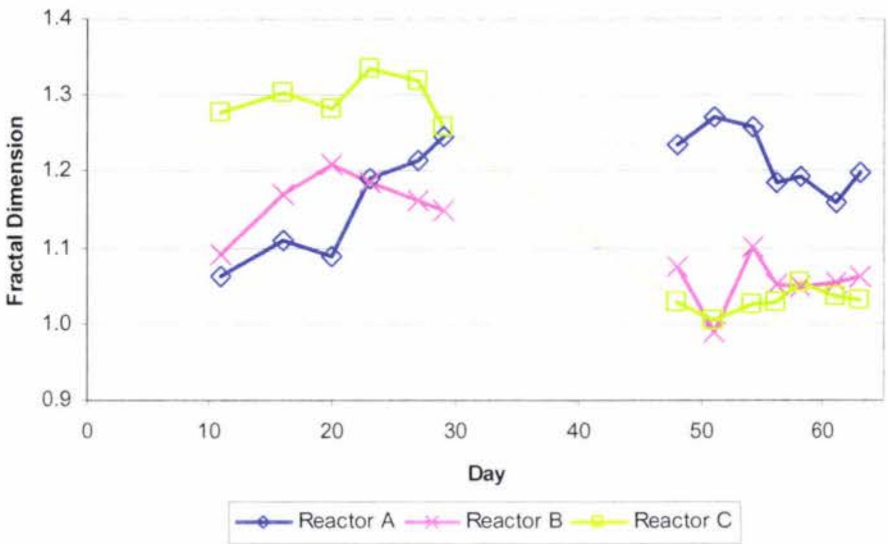


Figure 5.7 Fractal Dimension values for Reactor A (1 minute settling time), Reactor B (2 minute settling time) and Reactor C (15 minute settling time.

Figure 5.7 shows the fractal dimension calculated using the Zartarian *et al.*, (1997) area-perimeter relationship. While this relationship has similarities to that of the circularity, it has the advantage of being able to better elucidate the trends within the reactors. Figure 5.6 shows that although initially granules within Reactor A and B had a similar edge characteristic and both became more irregular over the first three weeks, Reactor A continued and maintained this increase in irregularity while the edge characteristic of Reactor B continued to become less irregular and rugged. Reactor C followed an opposite trend to that found in Reactor A, with an initially high fractal dimension, the granules within the reactor rapidly and suddenly become more uniform and less rugged, a finding not shown through the circularity data. Actual final dimension values were 1.20, 1.06 and 1.04 for the 1, 2 and 15 minute settling time reactors respectively.

5.3.6 Volume-Based Size Distribution

The mean size of particles found within each system was found to be distributed around a very low diameter (400 – 500 μm). Using the volume-based distribution it was possible to determine the 50th percentile particle size based on the particle biomass, with larger particles representing a significantly higher proportion of the total system biomass While some bias was created by the 200 μm cut-off the volume based distribution negates this as one granule with a diameter of 2 mm has the same volume as one billion particles with diameters of 200 μm. This means that larger particles are the distinguishing features within each reactor.

Analysis of the reactors was carried out to follow the evolution of the inoculum into granular particles. Figure 5.8, Figure 5.9 and Figure 5.10 are volume based cumulative graphs that show the evolution of granules within the 1 minute, 2 minute and 15 minute settling time reactors respectively. Comparison between these graphs shows the different effect that the settling time has on the size evolution and growth of the granules based on the volume of biomass.

Initially the effect of the higher levels of solids retention within Reactor B and Reactor C caused the populations of these two reactors to be larger than that within Reactor A. However larger particles were washed out of these two reactors by Day 16.

Within Reactor A the sizes of granules gradually increased over time and a greater percentage of the biomass is found within increasingly larger granules. One aspect of note is that due to the huge bias placed on larger granules the variation in the upper population range increases significantly as a greater number of larger granules became present within the system.

Within both Reactor B and Reactor C, a similar trend was noted. While some variation in the biomass distribution occurred only a slight increase in the median values was noted. This shows the despite the large difference in the settling times (2 minutes for Reactor B and 15 minutes for Reactor C) of these two systems, the biomass retained and grown had greater similarities than comparisons made between Reactor A (1 minute settling time) and Reactor B.

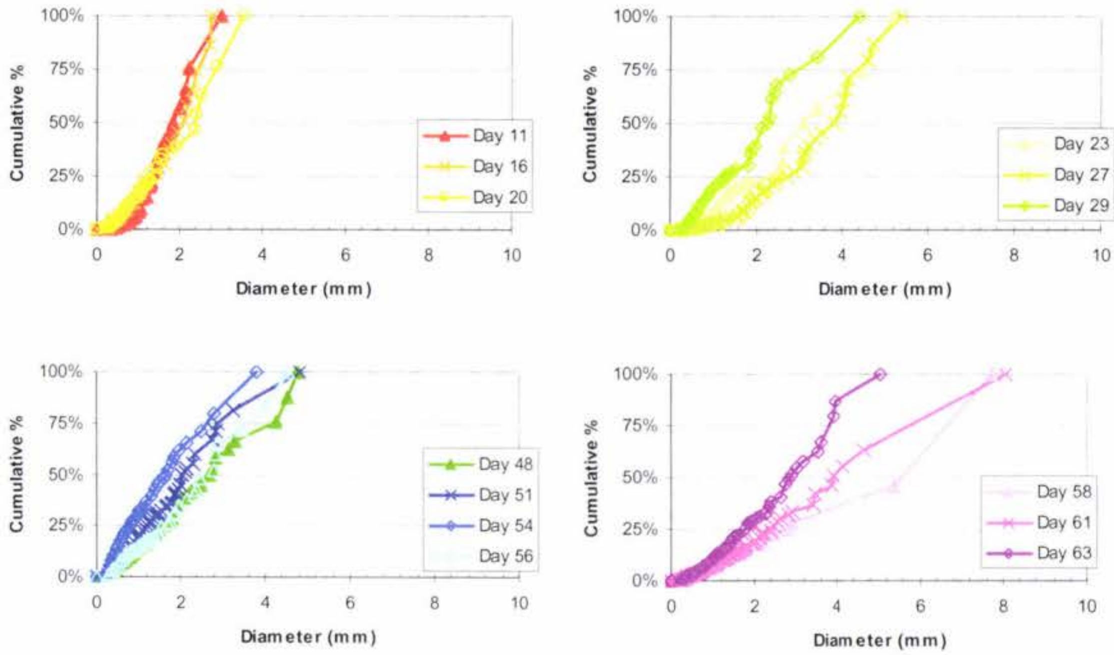


Figure 5.8 Volume based cumulative frequency graphs for the 1 minute settling time Reactor A.

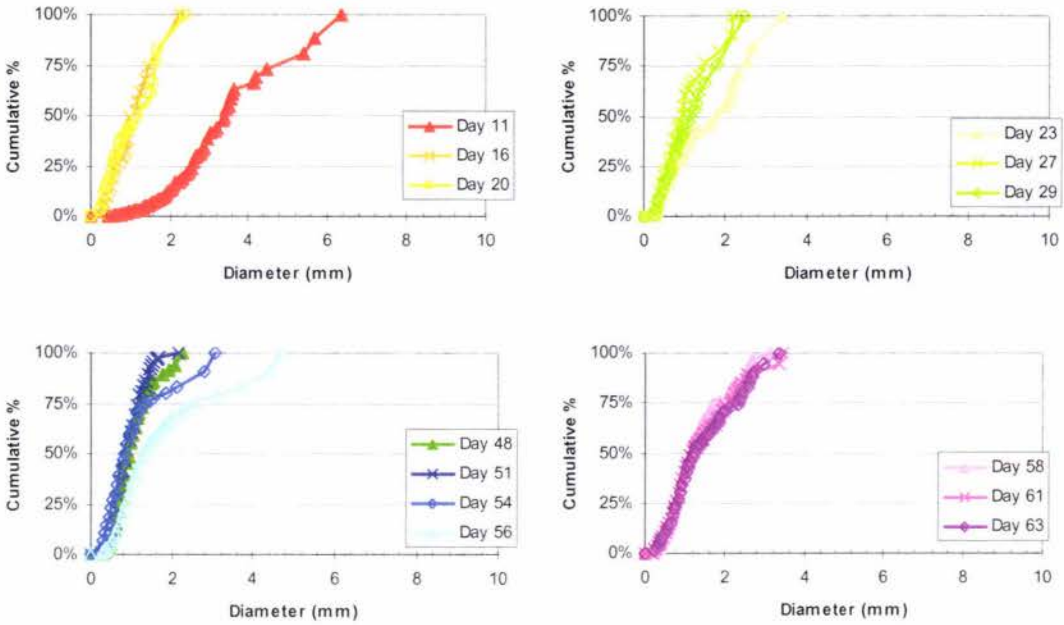


Figure 5.9 Volume based cumulative frequency graphs for the 2 minute settling time Reactor B.

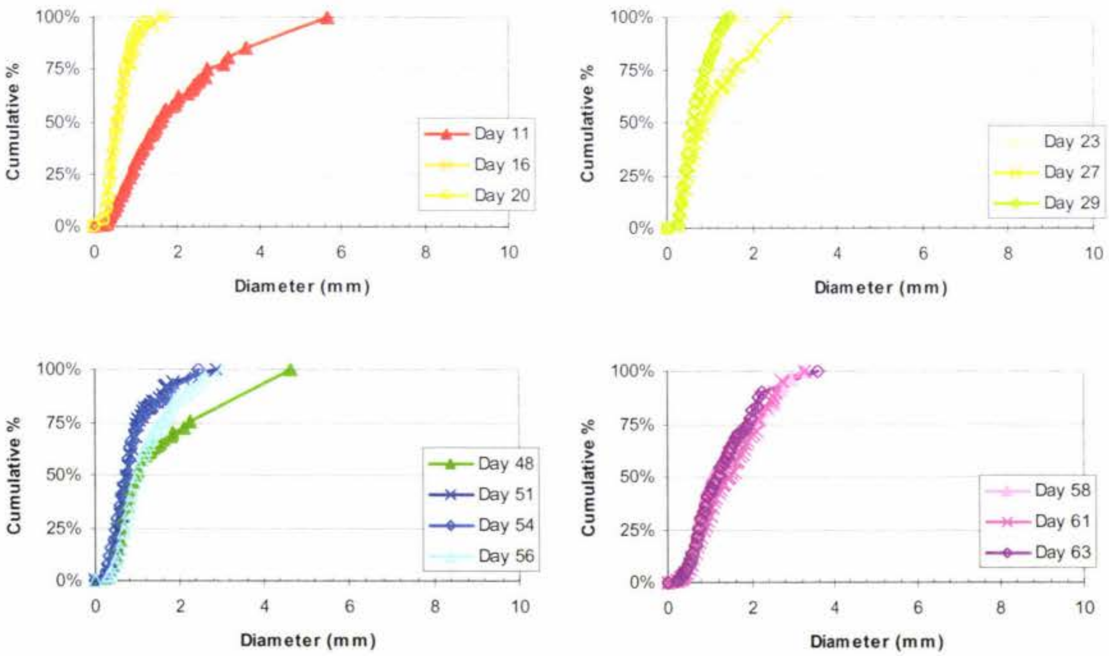


Figure 5.10 Volume based cumulative frequency graphs for the 15 minute settling time Reactor C.

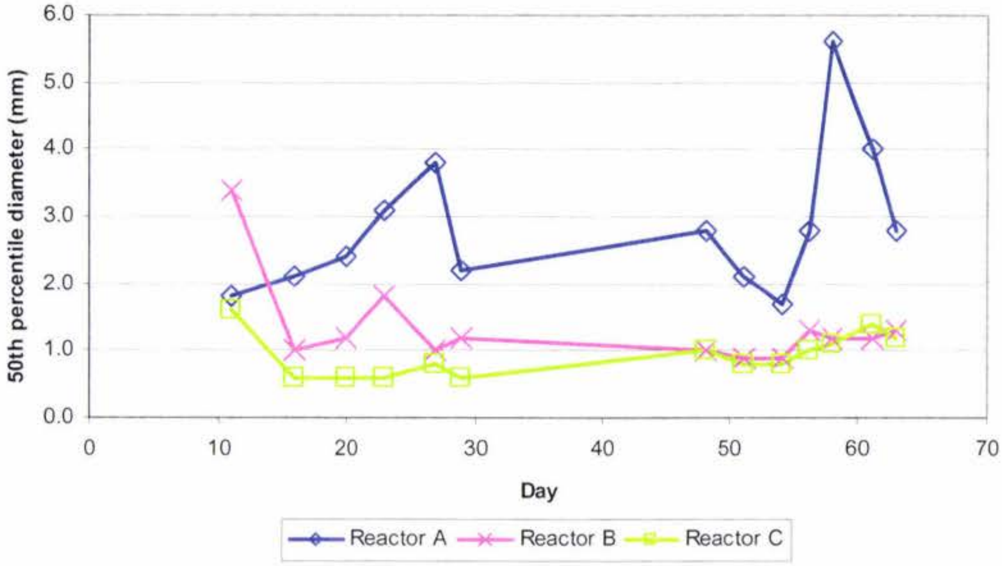


Figure 5.11 Volume based 50 % median diameters for the 1 minute (Reactor A), 2 minute (Reactor B) and 15 minute (Reactor C) settling time reactors.

Figure 5.11 details the change in the 50 % median for the volume-based distributions. Despite the fewer larger particles causing major deviations in the size distributions of Reactor A, the overall rate of increase of the median value is fairly constant and by Day 63 the median diameter was 2.9 mm. The similarities between Reactor B and Reactor C are clearly evident within the trends represented on this graph with both median populations reaching 1.2 mm diameters by Day 63. All three diameters fall within ranges outlined in literature (see *Literature Review Chapter 2* Table 2.5). Those granules generated within the 2 and 15 minute settling time reactors were however, generally, within the low range for granules generated within literature. Shown in Figure 5.12 are examples of these granules. The amount of non-granular biomass being retained with the longer settling time reactors (B and C) is also clearly shown.

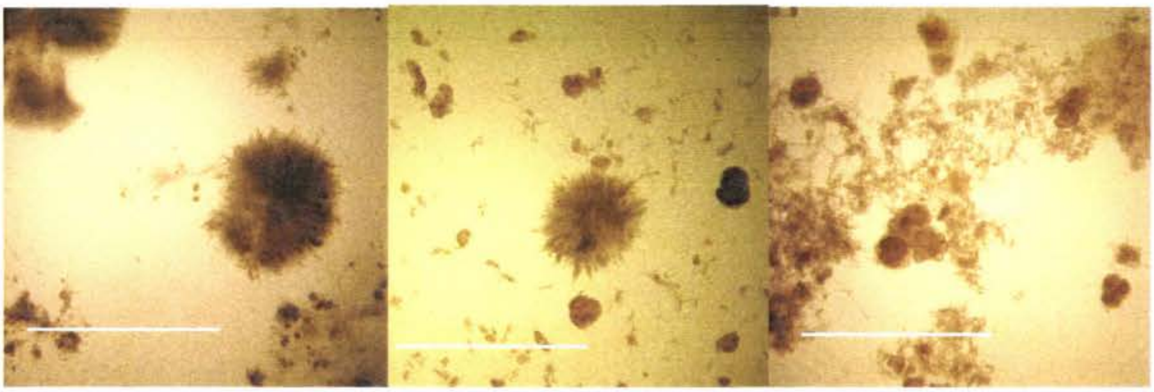


Figure 5.12: Typical Granules within Reactor A (left), Reactor B (middle) and Reactor C (right). Scale bar is 500 μm

5.3.7 Biochemical Characterisation

The ability of reactors to treat influent wastewater over all the settling times was an important feature. Granules are only useful for treatment processes if their carbon removal efficiencies remain high and the solids level released in the effluent low.

The rate of biomass accumulation was expected to be highest in Reactor C with its long settling time allowing the majority of biomass to settle, Reactor A was expected to gradually build up larger particles. Shown in Figure 5.13 are the solids concentrations taken within the mixed liquor and effluent over time. As expected Reactor A had a gradual build up of solids within the reactor mixed liquor and Reactor C had a much greater rate of accumulation. It is here that the effect of the potassium addition becomes evident. Almost immediately after potassium addition the solids accumulation increases significantly in all three reactors, doubling the biomass concentration with 3 weeks.

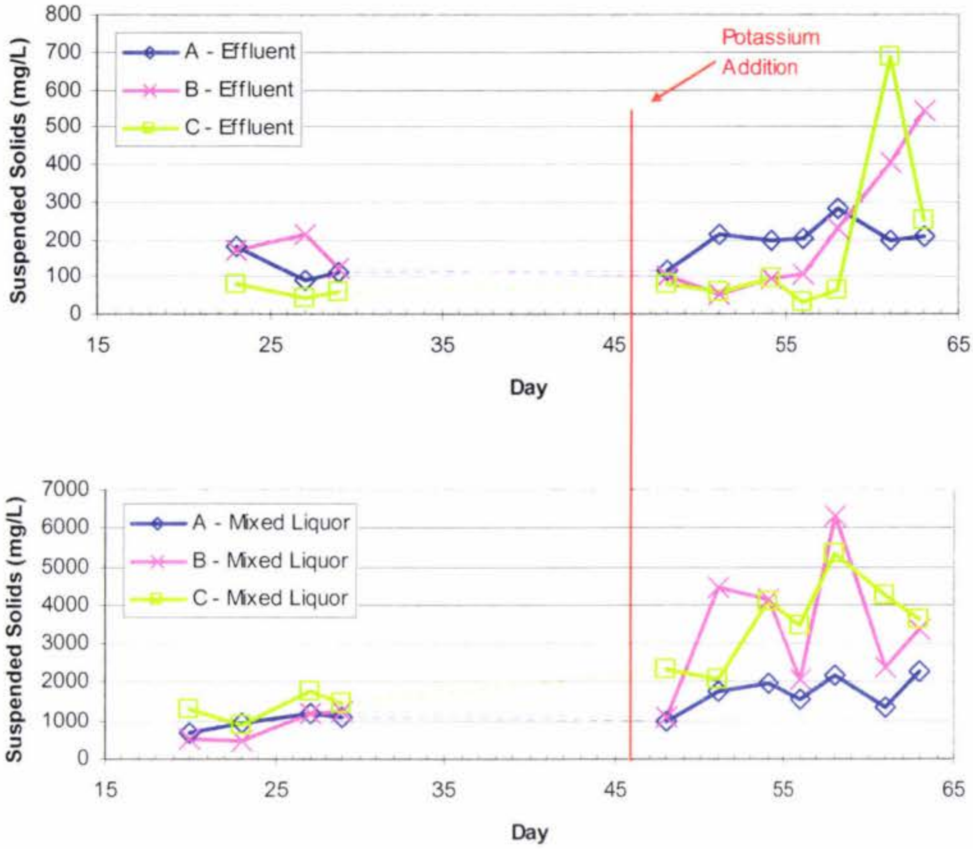


Figure 5.13 Total suspended solid concentrations for the effluent and mixed liquor.

The expected high level of washout within the short settling time reactors (A and B) occurred but an equilibrium between the washout and growth seemed to form and the amount of washout did not increase. However, the equilibrium was disturbed when potassium was added to the influent and the resultant increase in growth rate caused the amount of washout in all reactors to increase. Pre-addition the solids discharge was 100 mg/L for all three reactors. However, once potassium had been added the amount of solids being washed out of the reactor increased. In Reactor A this effluent solids level doubled to 200 mg/L and became increasingly erratic in the other two reactors as the settling of particles became hindered by the number of particles causing a variation in the measured levels of solids.

While there was some variation within the solids retention time (SRT), especially following the addition of potassium, it remained similar throughout the experiment. SRT averages were 4.4, 10.6 and 19.4 days for Reactor A, B and C respectively.

Using the chemical oxygen demand to determine the amount of soluble carbon in the mixed liquor and effluent (Figure 5.14), the treatment efficiencies for each reactor was calculated. It was found that within each reactor, treatment performance of over 95 % soluble COD removal was achieved following the addition of potassium and this is similar to levels of 96 % removal achieved by McSwain *et al.* (2004) in their granulation studies.

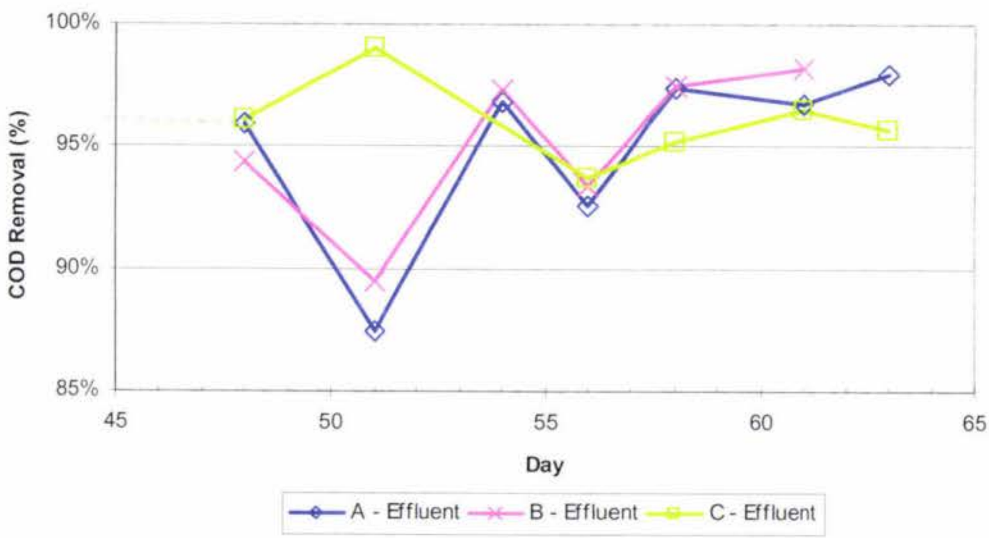


Figure 5.14 Chemical oxygen demand concentrations within the Reactor and Effluent

5.4 Summary

The effect of settling time was measured by the difference in characteristics of granules generated in three reactors with settling times of 1 minute, 2 minutes and 15 minutes.

Table 5.2 Summary Table of the Granule Characteristics on Day 63

	Reactor A *1 Minute Settling Time*	**Reactor B** *2 Minute Settling Time*	**Reactor C** *15 Minute Settling Time*
Volume-based diameter (mm)	2.8	1.2	1.2
Circularity	0.61	0.67	0.69
Fractal Dimension	1.20	1.06	1.03
Mixed Liquor Suspended Solids (mg/L)	2299	3358	3629
Effluent Suspended Solids (mg/L)	207	543	252
Effluent Soluble Carbon (mg/L)	6.0	7.7	15.1
Carbon Treatment (%)	96.3%	95.2%	95.3%

Shown in Table 5.2 is the Day 63 data taken from each reactor. The 1 minute settling time reactor was found to have significantly different physical characteristics from the two longer settling time reactors.

The settling time had a significant effect on the solids concentration within the reactors. The short settling time within Reactor A resulted in a much lower level of solids than in either of the other two reactors.

The treatment of carbon influent was identical within all reactors, showing that carbon removal is not limited by high or low settling times as shown previously by McSwain *et al.* (2004). A short settling time maintained advantages over longer settling times as it can reduced reactor down time and increases available reaction time. Further investigation into the effect of reduced hydraulic retention times is required to identify the extent of the advantages that this decrease could entail.

The visual progression and evolution of the biomass was documented and it became obvious that the short settling time reactor (A) had significantly larger particles than those found in either of the other two reactors. The form of these granules tended to be slightly less defined and more irregular in shape. Particles that settle faster were found to have an increased surface area which could allow for increased levels of reactivity.

The results from this work indicate that, between settling times of 1 and 2 minutes, there appears to be a critical point where granules within a reactor change from being large with high surface area to small with a more regular surface. This was determined through the consistent similarity between characteristics of the biomass within the 2 minute and 15 minutes settling time reactors. The cause of this could be attributed to the required minimum settling velocity associated within each reactor. Higher required settling velocity such as that found within Reactor A was found to yield granules of similar size in literature (Beun *et al.*, 1999).

Chapter 6: Effect of Nitrogen Deficiency

6.1 Introduction

The central aim of this thesis was to determine if it was possible to generate granules in systems where there was no or little supplementation of nitrogen, and nitrogen fixation is a likely process of obtaining balanced nutrients for cell growth. Available literature has shown that nitrogen fixing granular systems have not been investigated, highlighting the opportunity presented by the current work.

Typically, within nitrogen fixing systems, aeration is supplied by bubbling an air mixture up through the reactor (Chen *et al.*, 1984; Dennis *et al.*, 2004; Sheker *et al.*, 1993). Dennis *et al.* (2004) maintained the dissolved oxygen concentration about a setpoint of 2.1 mg/L or around 35 % of saturation. Low oxygen concentrations are usually maintained as some inhibition of nitrogenase, the nitrogen fixing enzyme, occurs at high concentrations (Gapes *et al.* 1999). This enzyme is responsible for the fixation of atmospheric nitrogen into a biochemically available form.

It has been proposed that due to the size of granular particles, there is potential for different areas of the granule to be populated with different microorganisms, with a range of different functionalities that could include aerobic, microaerophilic anoxic or anaerobic faculties. With such a heterogeneous population, the ability to remove compounds and utilise a wide spectrum of nutrients is increased. Concentration gradients into the granule mean that the central area has the potential to have low oxygen concentration and even anaerobic properties, as is shown in Figure 6.1.

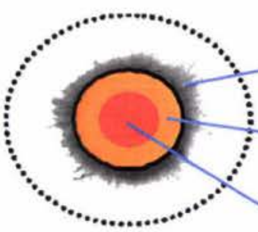


Figure 6.1 Aerobic granules have been found to have areas of different functionality.

In contrast to the requirements of nitrogen fixing systems, the generation of granules has been found to require high levels of aeration, which is necessary to provide sufficient turbulence (Liu *et al.*, 2003b; Tay *et al.*, 2001; Tsuneda *et al.*, 2003; Yang *et al.*, 2003). Generally this turbulence is provided through continuous aeration that effectively turns the reactor into an uplift bubble reactor. The extent of this turbulence requirement is further explored in Chapter 7. This chapter focuses on the differences that arose between identical granular systems grown on either high or low nitrogen concentration.

This apparent contradiction between nitrogen fixing systems and aerobic granular systems is an important concept to note. Around this contradiction it was hypothesised that under turbulent conditions, the need to protect the nitrogenase enzyme from elevated oxygen concentrations in the bulk liquid will cause the micro-organisms to adapt using different methods in order to survive. Part of this hypothesis was that nitrogen deficiency would cause the micro-organisms to cluster together to form a heterogeneous packed community. Another aspect was that protection of the enzyme would be through generation of high levels of EPS. Both of theses theories would result in a structure that would likely have the significant oxygen gradients, brought about by diffusion/reaction limitations (Gapes *et al.*, 2004). To test these hypotheses two reactors were run under highly turbulent aeration conditions, one reactor was supplemented with nitrogen (Reactor D) and the other without (Reactor E). The carbon :nitrogen ratios within the two reactors were 10.6:1.0 within the supplemented reactor and 552:1 within the deficient reactor.

6.2 The Reactors

Two reactors were run at 1 minute settling time to help select for larger particles (in accordance with the findings reported in Chapter 5) and at an 8 hour total cycle time. Reactors were run for 70 days (105 hydraulic retention times) and monitored over this period.

Prior experience with nitrogen fixing systems led to the expectation that growth would occur, despite a combination of high oxygen concentration and lack of nitrogen supplementation (Reactor E). It was thought that any granules that would grow within Reactor E would be larger and denser than those within Reactor D as the different species of micro-organisms would colonise in areas where conditions were more favourable as well as produce increased amounts of extra-cellular polysaccharides (EPS) which help reduce diffusion into the granule.

6.3 General Observations

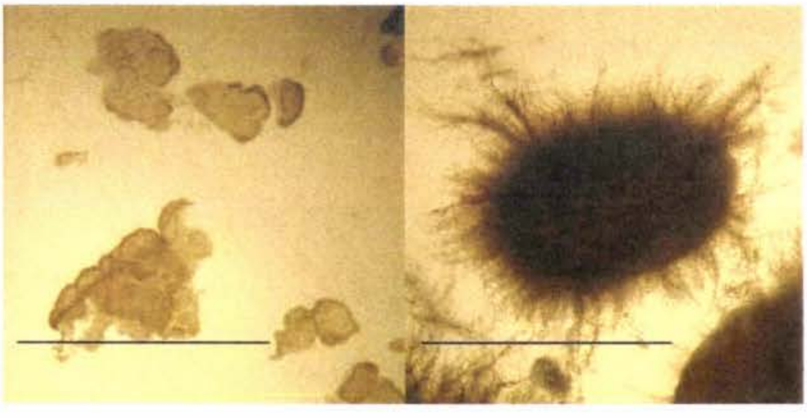


Figure 6.2 Stereomicroscope biomass pictures from the nitrogen supplemented (Reactor D –Left) and the nitrogen deficient (Reactor E – Right) systems. Scale bar denotes 500 μm.

Granules were found to develop within both the supplemented and deficient reactors. This shows that Reactor E was able to generate granules despite the lack of nitrogen. The granules grown within the two reactors were found to be very different in appearance as is shown in Figure 6.2. Those granules grown within the reactor that was supplemented with nitrogen (Reactor D) were smaller and more compact in appearance than those grown under nitrogen deficient conditions (Reactor E). This size differential was reflected in the cumulative volumetric size distributions shown in Figure 6.6.

6.4 Population Characterisation

Using the T-RFLP method, a qualitative analysis of the population within each reactor was carried out (Figure 6.3). This analysis found that the populations within Reactor D (nitrogen supplemented) were less diverse than those found within Reactor E (nitrogen deficient) but many of the populations found within D are also found within E. The increased diversity could be due to the requirement of the deficient reactor to develop a more symbiotic community where each group of organisms is responsible for a specific purpose, such as generate available nitrogen through fixation or increased production of EPS compounds. Within the graph, the different organism groups are represented by number bands, with closer numbers associated with similar organism groups.

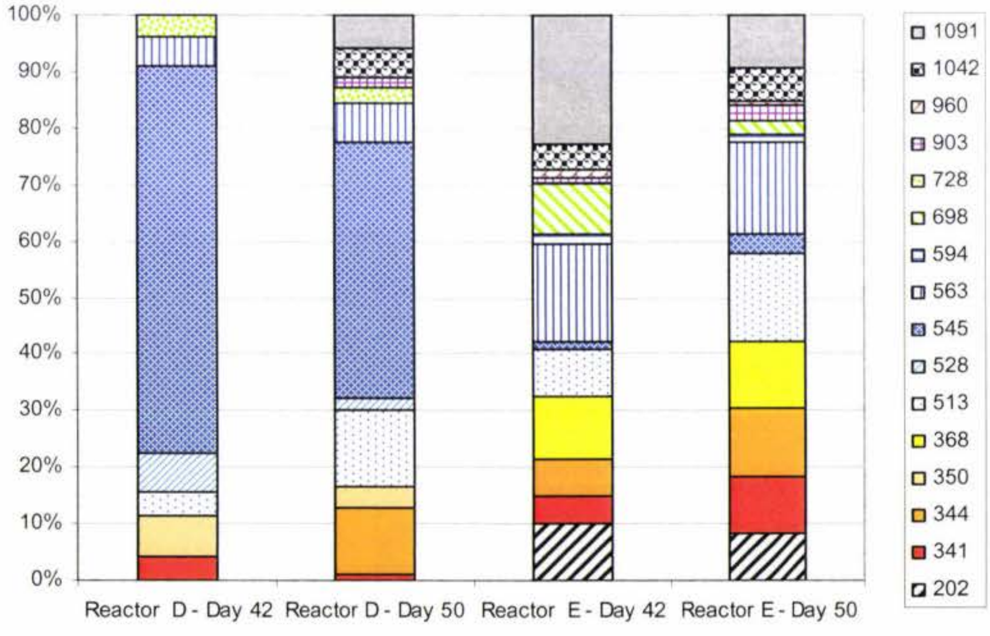


Figure 6.3 T-RFLP Population study. Bands identify different terminal that determine population fractions within the reactors (as denoted in the legend).

Through changes in only the nitrogen concentration the populations within the reactor were found to differ. Also, characteristics of the granules generated within each reactor were found to differ dramatically. Previous studies have found that changes in influent carbon substrate (Moy *et al.*, 2002) substrate concentration (Tay *et al.*, 2004; Yang *et al.*, 2004b) and other components (Yang *et al.*, 2004a; Yang *et al.*, 2004b; Yu *et al.*, 2001) have had influence on the formation and growth of granules. The effect on the actual biota of a system has not been comprehensively investigated.

6.5 Nitrogen Fixation

While growth occurred within the nitrogen deficient reactor it was assumed that the required nitrogen was obtained through nitrogen fixation. In an effort to confirm the presence of nitrogen fixation, acetylene reduction assays were carried out. The gas chromatograms from these tests are shown in Figure 6.4. The nitrogen supplemented system (Reactor D) has a peak for acetylene (t = 3.35 min) but the peak for ethylene (t = 2.4 min) is absent. This ethylene peak is present in the sample for the nitrogen deficient system (Reactor E), showing the acetylene is being reduced to ethylene within this reactor.

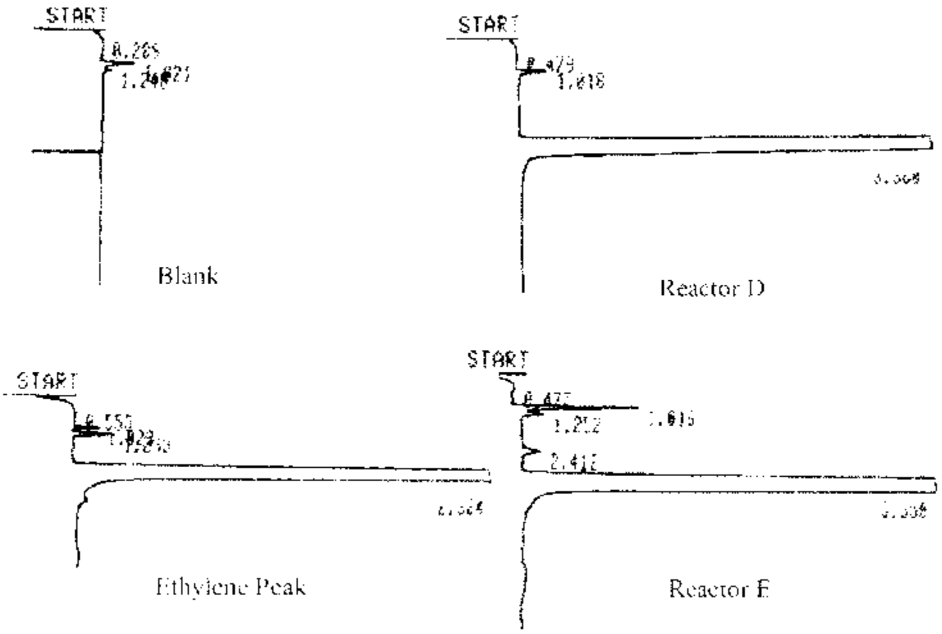


Figure 6.4 Acetylene Reduction Chromatograms. The ethylene standard shows the peak for ethylene (product of acetylene reduction) and this peak is found only in the results from the nitrogen deficient system (Reactor E).

6.5.1 Image Analysis

Shown below in Figure 6.5 is mixed liquor comparison from the two reactors. The granules within both reactors are clearly evident and different from each other. Within the nitrogen supplemented system, Reactor D, the granules are small and tightly defined while within the nitrogen deficient system, Reactor E, the granules are larger and appear fluffy.

Figure 6.5: Mixed Liquors - left Reactor D, right Reactor E (taken Day 72)

Analyses were carried out on data collected on Day 61 of the study. Image analysis revealed that half the volume-based mass Reactor D had a diameter of over 2.2 mm (Figure 6.6). Within Reactor E this size increased to 6.1 mm. Thus the average diameter of particles within the nitrogen fixing reactor was three times greater than that within the reactor with nitrogen supplementation. This characteristic supports the hypothesis of increased growth in nitrogen fixing systems for creation of dramatic diffusion gradients within the granule. As the reactors are continually seeding new granules it is hypothesised that the initial stages of granulation are similar in both systems. The similarities between the systems end when those granules within the nitrogen deficient reactor reach a certain threshold size, and they are able to provide sufficient zones of low oxygen concentration for the nitrogenase enzyme to work more efficiently. This provides the granules in the nitrogen deficient reactor with a predilection for increased size and this is reflected in the population distribution.

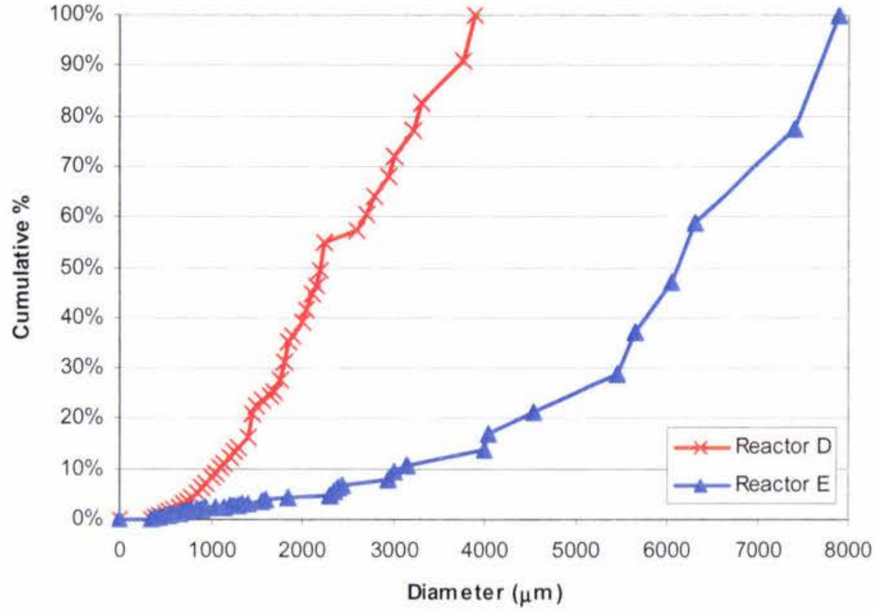


Figure 6.6 Volume based cumulative distributions for the nitrogen supplemented (Reactor D) and nitrogen fixing (Reactor E) systems, on Day 61.

The other visually obvious difference between the two reactors is the edge characteristic and filamentous nature of the granules. The nitrogen supplemented granules (Reactor D) had a smooth outer edge and they were irregularly shaped. Those granules within the nitrogen deficient system (Reactor E) were highly filamentous with many tendril-like outgrowths and significantly more 'rugged' than those in Reactor D. Through image analysis, characterisation of the edge of the granules within the two systems was carried out and is shown in Table 6.1. The circularity of granules within both systems was found to be similar with a value of 0.41 for the nitrogen supplemented system and 0.45 for the deficient system. However the difference in the reactor fractal dimension values is an interesting comparison. Supporting the visual observations, the nitrogen deficient granules have higher fractal values than those found for the nitrogen supplemented granules.

The investigation into the characteristics of the bulk population of both of the reactors revealed that despite the lack of nitrogen addition to Reactor E the granules that developed were much larger than those that formed under nitrogen sufficient conditions within Reactor D. These granules from Reactor E also had a much more rugged and filamentous surface than the relatively smooth one generated within Reactor D.

Table 6.1 Edge characteristics within the nitrogen supplemented (Reactor D) and nitrogen deficient (Reactor E) systems, on Day 61.

	Reactor D	Reactor E
Circularity	0.413	0.451
Fractal Dimension	1.057	1.139

6.6 Individual Granule Analysis

Further investigation into the effects that running systems under nitrogen deficient conditions had on aerobic granules was carried out through analysis on individual granules. Fifteen granules from both systems were taken on Day 72 and analysed. These analyses were carried out to determine the image based physical characteristics, settling velocities and densities of each granule.

From the pictures taken for this analysis there was a clear difference between granules grown in each reactor and a representative granule is shown in Figure 6.7. The granules within the nitrogen supplemented reactor (Reactor D) were much smaller than those in the nitrogen fixing reactor (Reactor E).

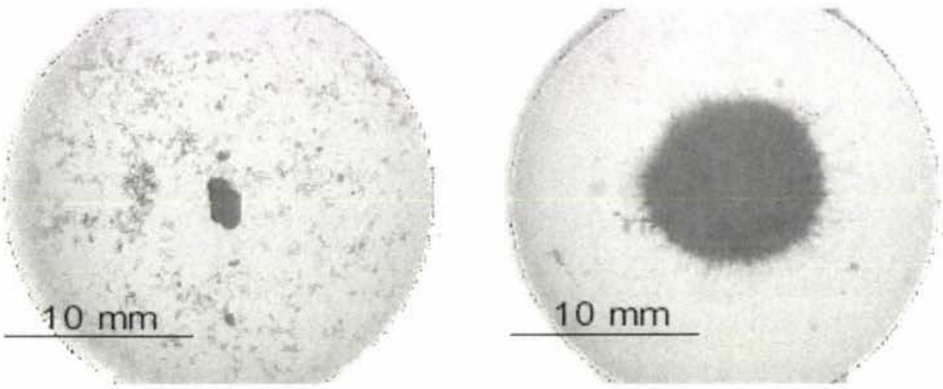


Figure 6.7 Individual granule comparisons. Left granule from the nitrogen supplemented system (Reactor D), right granule from the nitrogen deficient system (Reactor E).

Table 6.2 shows the characteristics of the granules analysed from the two systems. From this table it is reiterated that the particles within the nitrogen supplemented system (Reactor D) are much smaller, with a mean equivalent radius of 2.0 mm, than those granules in the nitrogen deficient system (Reactor E) which had an equivalent diameter of 7.0 mm. This significant difference in size is also shown in the means of the area, perimeter and Feret's diameter for each of the Reactors. These differences place even further emphasis on the difference in size between the two reactors.

Table 6.2 Reactor D (Nitrogen Supplemented) and Reactor E (Nitrogen Deficient) Granule Characteristics

	Reactor D		**Reactor E**	
Characteristic	**mean**	***std dev.***	**mean**	***std dev.***
Area (cm^2)	0.03	*0.01*	0.39	*0.12*
Perimeter (cm)	0.82	*0.17*	4.97	*1.00*
Circularity	0.61	*0.12*	0.21	*0.08*
Ferets Diameter (mm)	2.53	*0.45*	8.29	*1.32*
Equivalent Diameter (mm)	2.01	*0.32*	7.00	*1.05*
Fractal Dimension	1.58	*0.04*	1.62	*0.03*
Settling Velocity (cm/s)	0.88	*0.15*	1.42	*0.23*
Density (kg/m^3)	1019	*10*	1002	*1*

Granules found within the nitrogen supplemented system fit within the expected size distributions from literature of 0.25 mm to 6.50 mm (Beun *et al.*, 2001; Moy *et al.*, 2002; Su and Yu, 2004; Tay *et al.*, 2001; Yang *et al.*, 2004b). Those granules within the nitrogen deficient reactor however are much larger than those in reported literature with the largest maximum granule size of 6.5 mm, by Etterer and Wilderer (2001). And even this literature finding was for a glucose-fed study which tended towards having larger sized particles than those where acetate was the substrate (Moy *et al.*, 2002; Tay *et al.*, 2001).

At 1002 kg/m^3, the density of nitrogen deficient granules was significantly lower than that of the nitrogen supplemented granules, 1019 kg/m^3. With higher density the granules have better settleability and a higher concentration of biomass. The lower density within the larger deficient granules could be caused by a greater proportion of voids or significant gel-like EPS with high water holding capacity, but little density difference to that of water.

As shown, the granules within nitrogen supplemented system (Reactor D) are much more circular than granules within the nitrogen deficient system (Reactor E), with the stereomicroscope images better representing these differences than those taken using the digital camera. For the bulk study, using images acquired with digital camera magnification and resolution, the mean circularity was 0.413 and 0.451 for the nitrogen supplemented and deficient reactors respectively. This contrasts with values of 0.61 and 0.21 for the individual granules as viewed under a stereo microscope.

Similar discrepancies to those found within the circularity values were found in the investigation into the fractal dimension of granules. The use of the ImageJ plug-in FracLac on the stereomicroscope pictures resulted in a significantly different value than that obtained through the perimeter-area relationship, as proposed by Zartarian *et al.* (1997), and used on the digital camera images.

The mean fractal dimension, calculated from each individual image, using FracLac was found to be 1.58 for Reactor D and 1.62 for Reactor E. The relationship between circularity and fractal dimension give a quantified idea of the granular edge characteristics. Due to the more filamentous nature of nitrogen deficient granules the circularity (as is determined by a perimeter:area relationship) is considerably lower than that of granules generated within the nitrogen supplemented system. The similarity of fractal dimension shows that the edges of granules within both reactors were in fact very similar. This shows that a real difference between granules is in the development of the tendril-like filaments on the nitrogen deficient granules from Reactor E.

The differences between the stereomicroscope and digital camera pictures and data meant that the two methods of determining the fractal dimension values were not interchangeable. These differences however are explainable. The increased resolution of the stereomicroscope enabled the analysis programme to pick out the more minute details on the edges of the granules. Hairy tendrils that would were blurred and lost within the digital camera pictures were identifiable within the stereomicroscope pictures but further emphasised the differences between granules in both reactors as was shown in Figure 6.7. This fits in well with the previous 'coastline' analogy for describing fractal dimensions, it can be assumed that even great magnification of the edge would allow an even more accurate surface characterisation.

6.7 Settleability

Granules within the nitrogen deficient system (Reactor E) were found to settle much faster, at 1.42 cm/s, than granules within the nitrogen supplemented system (Reactor D) at 0.88 cm/s. Literature found that settling velocities of granular systems ranged between 0.7 and 3.1 cm/s (Etterer and Wilderer, 2001; Jang *et al.*, 2003; Morgenroth *et al.*, 1997; Moy *et al.*, 2002; Su and Yu, 2005; Tay *et al.*, 2001; Wang *et al.*, 2004; Zhu and Wilderer 2003). Interestingly, while the granule density was significantly lower in the nitrogen fixing system (Table 6.2), this characteristic was offset in the settling rate comparison by the dramatic difference in aggregate diameter (see Stokes terminal settling velocity equation in *Section 2.3.3.1*).

The packing of granules within the system as it settles can be represented by the sludge volume index (SVI). The SVI is shown in the Figure 6.8. The average SVI for both reactors was good (< 150 mL/g). That the SVI of granules within Reactor E is 50 mL/g is likely caused by the increased settling velocity and particle size over the smaller granules found in Reactor D. This result is highly significant, as flocculent nitrogen deficient systems often display signs of bulking and have SVI values > 200 mL/g (Dennis *et al.*, 2005). Clearly, the aim of developing a nitrogen fixing granular system in order to improve sludge settleability of this treatment technology has proven successful.

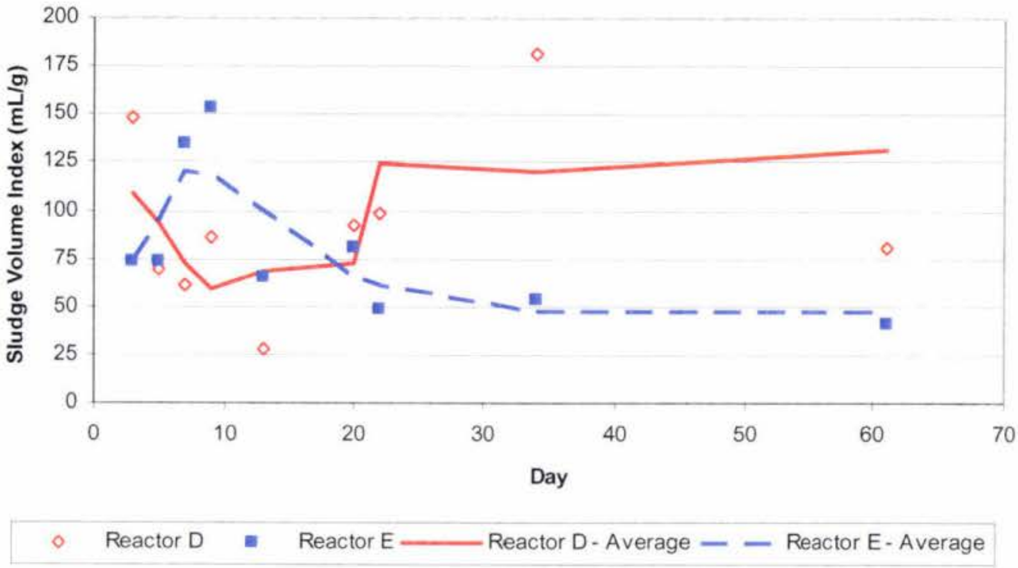


Figure 6.8 Sludge Volume Index of solids found within Reactor D (Nitrogen Supplemented) and Reactor E (Nitrogen Deficient)

6.8 Comparison of Nitrogen Supplemented and Deficient Values

A correlative analysis was undertaken in an attempt to quantify the effect that the differing nitrogen concentrations had on these characteristics. Using the Total Kjeldahl Nitrogen concentrations of 50 mg/L (Reactor D) and 5 mg/L (Reactor E) as values for the concentration of nitrogen added to each reactor, the correlations shown in Table 6.3 were found. Image data was compiled from the data obtained investigating the bulk characteristics of the reactor systems.

Table 6.3 Correlation for Reactor D and Reactor E

	N Suppl	*Density*	*Fractal*	*Area*	*Perim.*	*Circ.*	*Feret*	*S.Velocity*
N Suppl	1.00							
Density	0.82	1.00						
Fractal	-0.47	-0.65	1.00					
Area	-0.89	-0.74	0.39	1.00				
Perim.	-0.94	-0.78	0.52	0.94	1.00			
Circ.	0.91	0.88	-0.69	-0.83	-0.94	1.00		
Feret	-0.94	-0.81	0.46	0.99	0.96	-0.88	1.00	
S. Velocity	-0.83	-0.73	0.33	0.87	0.84	-0.79	0.88	1.00

This table shows that nitrogen supplementation had an interesting impact on all the granule characteristics analysed, except for the aforementioned fractal dimension. As predicted the nitrogen deficient reactor had granules that were larger than those in the nitrogen fed reactor. Other differences show that although the granules in Reactor D were denser than those found in Reactor E they did not settle faster. This can be attributed back through to Stoke's settling law as the nitrogen deficient granules are larger than those grown within the reactor run with supplementation. This is an attribute which is favourable for faster settling systems.

6.9 Treatment Characteristics

A comparison of the treatment efficiencies of the two reactors was carried out to determine the carbon removal occurring and also the levels of nitrogen and phosphorus in the effluent.

6.9.1 Reactor Solids Level

The biomass concentration within each reactor was measured as the total suspended solids and this was used as an indication of the development and change of the populations within each reactor (Figure 6.9). It was found that the TSS for Reactor D (nitrogen supplemented) remained constant, with some fluctuations, over the length of the experiment with a concentration between 1.0 and 2.0 g/L. Within Reactor E (nitrogen deficient) the changes in biomass concentration were much more significant. Within Reactor E, over the initial 10 days of operation the TSS halved from a concentration of over 2 g/L down to a minimum value of 1 g/L. From Day 10 the TSS value continued to increase as the granule population within the reactor continued to mature and grow finally achieving a biomass concentration of 4.0 gTSS/L. This accumulation of biomass can partially be attributed to the increased size and settling velocity within the deficient reactor meaning fewer granules are washed out.

Comparison of the value for the nitrogen supplemented reactor and values from literature (Morgenroth *et al.*, 1997; Tay *et al.*, 2004a) show that at an organic loading rate of 3.2 kgCOD/(m^3.day) should produce significantly higher levels of biomass concentration. At a loading rate of 4.0 kgCOD/(m^3.day) and 80 days of operation, Tay *et al.* (2004) produced a reactor with 12 g/L suspended solids.

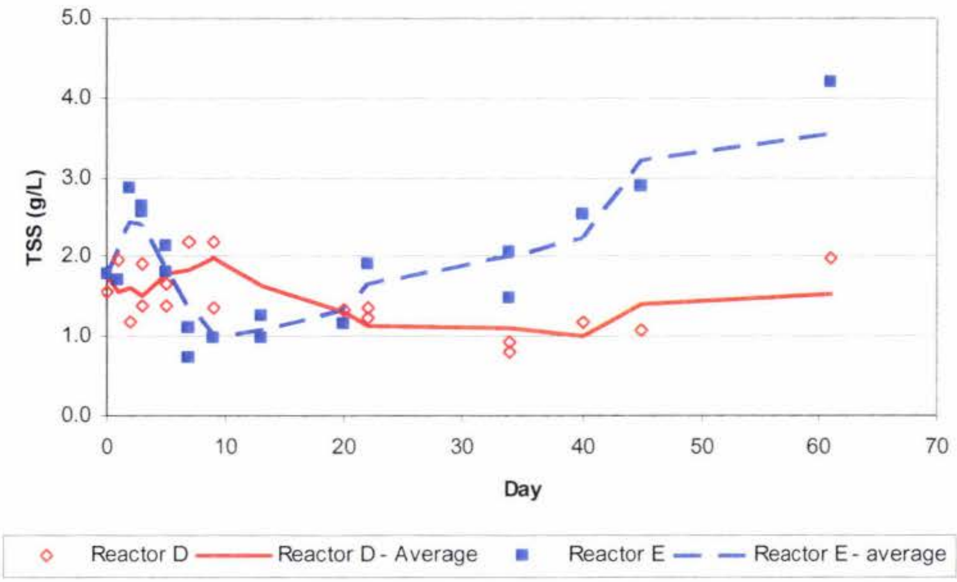


Figure 6.9 Total mixed liquor suspended solids found within Reactor D and Reactor E

Suspended solid values taken from the effluent show that despite the high mixed liquor biomass concentration within Reactor E the solids removal efficiencies were not compromised (Figure 6.10). Washout within the nitrogen supplemented reactor was found to be higher however, the final trend demonstrated is a decrease in suspended solids, this shows the nitrogen fixing systems may have some advantage over supplemented systems due to lower effluents solids concentrations.

In what appears to be somewhat of a mark of granular systems, due to the selective settling times the effluent solids concentration has been found to be higher than for standard SBR systems. Beun *et al.* (2002) found that for the first 50 days of operation the concentration of solids within the effluent was erratic and varied between 0.15 and 0.35 g/L. The same study found that after 140 days of operation this solids level dropped down to 0.07 g/L. McSwain *et al.* (2004) found that the effluent solids concentration, within the two-minute settling time reactor run in their study, was 0.169 ± 0.095 g/L between days 120 to 220. These studies reveal that the nitrogen deficient reactor from this current work has achieved an effluent solids concentration comparable to that of another reactor operated for over twice the operational time. The effluent solids level within the nitrogen supplemented reactor, shows evidence of similarity with these studies.

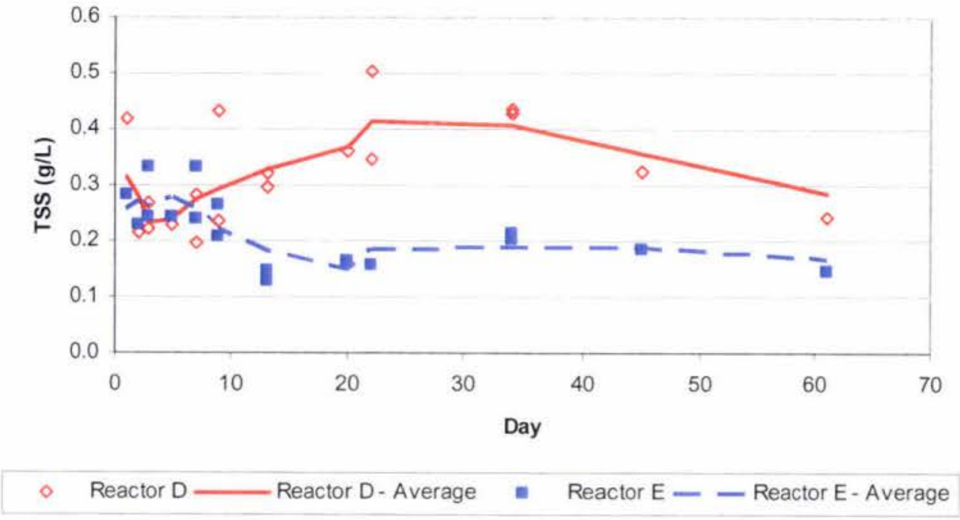


Figure 6.10 Total suspended solids found within effluent of Reactor D (Nitrogen Supplemented) and Reactor E (Nitrogen Deficient)

6.9.2 Carbon Removal

Figure 6.11 shows the soluble organic carbon concentration within the effluent from both reactors. During the first 10 days following start-up, Reactor D (nitrogen supplemented) had a carbon concentration within its effluent below 10 mg/L while Reactor E (nitrogen deficient) had a concentration between 10 and 15 mg/L. Whilst Reactor E was capable of maintaining this concentration the concentration within Reactor D continues to increase. There is a lack of data following Day 40 when the solids within the effluent began to decrease, thus it cannot be confirmed whether this decrease is also reflected in the soluble fraction of the effluent.

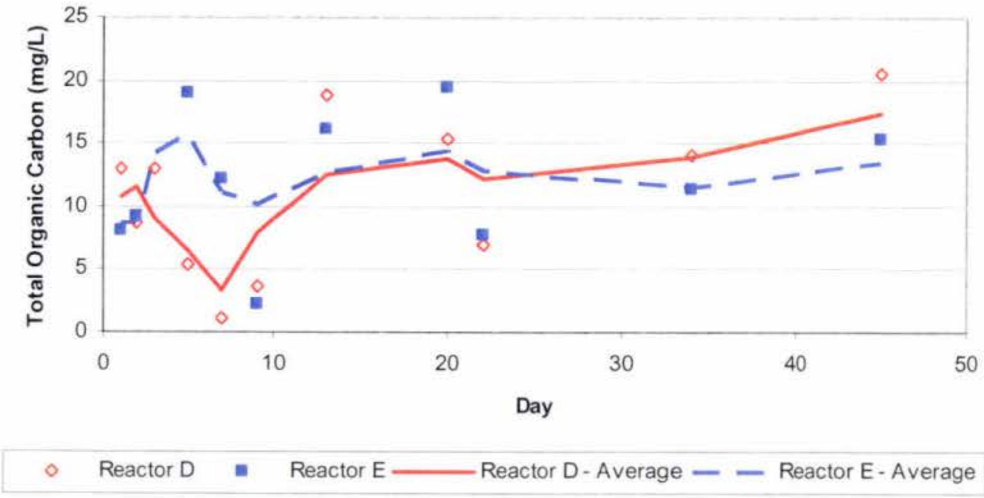


Figure 6.11 Organic carbon concentrations within the effluent for Reactor D (Nitrogen Supplemented) and Reactor E (Nitrogen Deficient)

With a total organic carbon concentration of the feed at 500 mg/L it was possible to calculate the treatment efficiency of both reactors and this is shown in Figure 6.12. Throughout the study both reactors were able to treat above 95 % efficiency, with the nitrogen deficient reactor removing between 97 – 98 % of the influent carbon. This is an important characteristic as it shows that granules systems, and in particular nitrogen deficient systems, are capable of treating synthetic wastewater at high efficiencies. The declining efficiency of the nitrogen supplemented reactor is of concern, only further investigation will be able to determine the extent of the drop in efficiency.

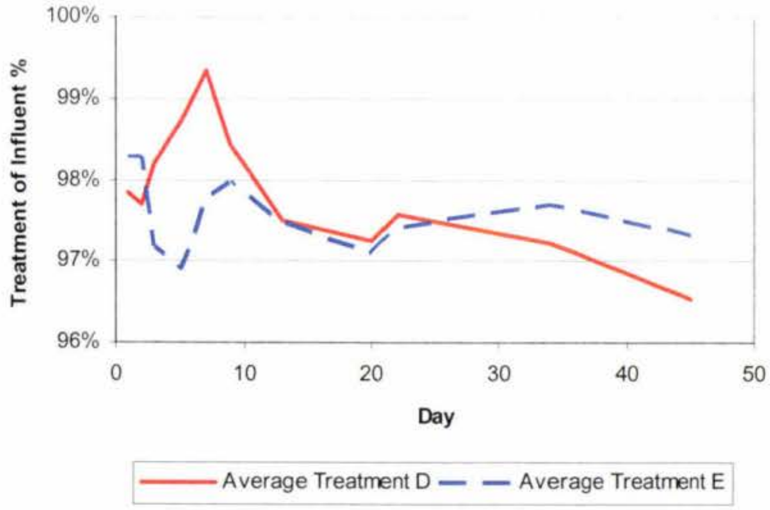


Figure 6.12 Treatment Efficiencies for Reactor D (Nitrogen Supplemented) and Reactor E (Nitrogen Deficient)

The dissolved oxygen (DO) concentrations within each reactor were recorded. These values were of particular interest within the nitrogen deficient reactor due to the inhibition of the nitrogen fixing enzyme at high oxygen concentrations. During the settle, decant and feed stages the concentration within both reactors continued to drop as the biomass within the reactor continued to use the available oxygen and the bubbling of air through the reactor was stopped. At the end of the feed phase the aeration would restart and the oxygen concentration would gradually increase back to saturation, with particular increases observed at the end of the feast period (elimination of the acetate from the reactor liquid). Recovery profiles for five days over the course of the study are shown in Figure 6.13. During the early stages of the study the oxygen concentration within Reactor D (nitrogen supplemented) decreased to 5.5 mg/L (70 % of saturation) during the feast phase. By Day 35 however the extent of this drop had decreased, with a minimum concentration of 6.0 – 6.5 mg/L. In comparison to those recovery curves, Reactor E (nitrogen deficient) maintained a drop to a minimum concentration of between 6.0 and 7.0 mg/L throughout the entire study. Despite this high DO level nitrogen fixation was still found to occur as shown by the nitrogen fixing assays (Figure 6.4).

Drops in the DO concentrations can be directly related back to the biomass concentration within reactors (as shown previously in Figure 6.8). As the biomass increased in concentration the drop in DO was more pronounced. Following the biomass decrease in the nitrogen supplemented system (Reactor D) between Day 10 and Day 20 the DO drop decreases accordingly. Within the nitrogen fixing system (Reactor E) the effect is not as pronounced but closer inspection shows this relationship is still present.

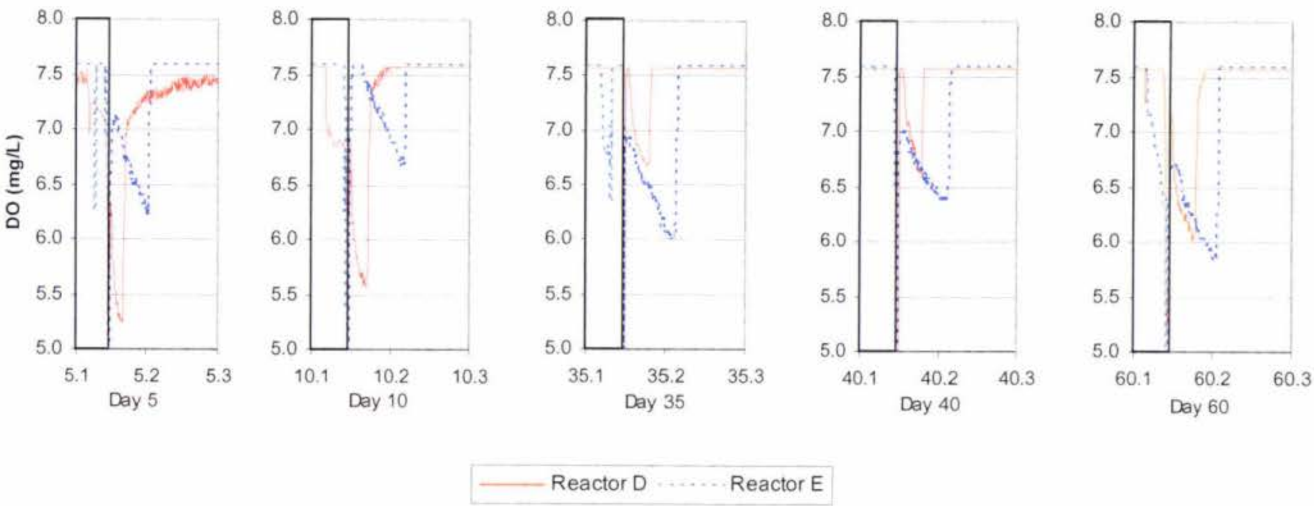


Figure 6.13 Dissolved Oxygen Profiles within the Reactor D (Nitrogen Supplemented) and Reactor E (Nitrogen Deficient) systems. The shaded areas denote timing of decant and feed periods.

Shown in Figure 6.14 are the specific oxygen uptake rate (sOUR) profiles that further detail the effect that the increasing biomass has on the reaction rate, and the consumption of oxygen, within the nitrogen supplemented and nitrogen deficient systems. These graphs show that the period where oxygen is consumed in the feast phase is half as long within the nitrogen supplemented system (Reactor D) as it is within the nitrogen deficient system (Reactor E). This was consistent throughout the study despite the increase in biomass within both systems. The increase in biomass does reduce the maximum sOUR that occurred within both systems and this can be attributed to the finite carbon concentrations added each cycle. The calculation does have some inaccuracies: it assumes that all the biomass within the system is active but with large granules and EPS within the reactors a significant proportion will not be. The sOUR calculation also assumes that the viscosity within the systems remains constant but this will vary depending on the constituents dissolved within the reactors.

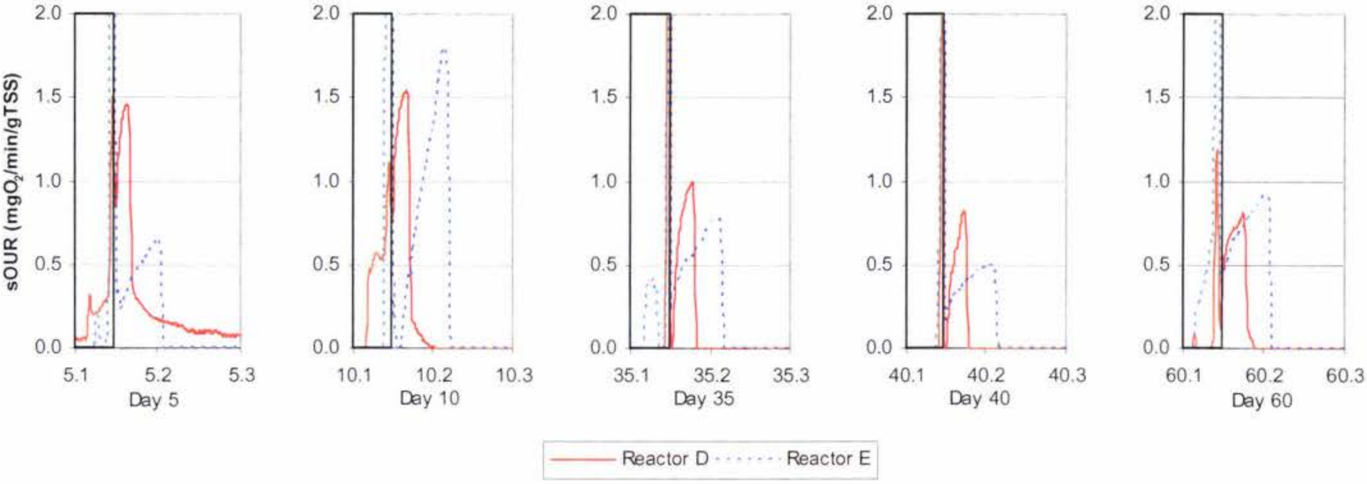


Figure 6.14 Specific Oxygen Uptake Rate Profiles within Reactor D (Nitrogen Supplemented) and Reactor E (Nitrogen Deficient) systems. The shaded areas denote timing of decant and feed periods.

The K_La_{O2} reaction constant for these reactors was 1.06 /min with a reciprocal time interval of 56.6 seconds. However, the response time of the DO probe was calculated to be 78 seconds. This represents a sensor lag time of over 30 %. Without the ability to measure the DO concentration within the reactor at intervals less than the inverse K_La_{O2} constant these values can only be a qualitative measure of the sOUR.

6.9.3 Nitrogen Treatment

The nitrogen, in the forms of Kjeldahl, total (TKN) and dissolved (DKN), nitrogen oxides (NO_x) and ammonium (NH_4^+) was measured in the feed and effluent streams from both reactors.

The feed concentrations of nitrogen are shown in Table 6.4. The influent being fed into the nitrogen deficient reactor had concentrations of nitrogen compounds that are roughly 10 % of that being added to Reactor D.

Table 6.4 Nitrogen Compound Influent Concentrations for Reactor D (Nitrogen Supplemented) and Reactor E (Nitrogen Deficient)

Component	Reactor D (mg/L)	Reactor E (mg/L)
NO_x	0.03	0.03
TKN	52.4	6.8
DKN	45.0	4.8

Investigation of the effluent found that nitrogen oxides within the effluent never exceeded 0.04 mg/L, revealing that significant amounts are not generated. As expected the level of ammonium within the effluent was higher within Reactor D, between 2.0 and 3.0 mg/L, than Reactor E, which never increased past 0.5 mg/L. Both reactors show some overall removal of nitrogen in this form.

Effluent Kjeldahl nitrogen concentrations are shown in Figure 6.15. TKN levels within the effluent of the nitrogen supplemented reactor were found to maintain around 50 mg/L while the levels within the other reactor had slightly more variation between 20 to 30 mg/L. The levels of TKN within the effluents of both reactors are subject to the effluent solids concentration. As a portion of biomass is nitrogen, 12.2 g of nitrogen for every 100 g of cell biomass (Metcalf and Eddy, 2003), this describes a significant portion of the TKN in the effluent of both reactors. From the biomass suspended solids within the effluent, 62% and 74% of the TKN within the supplemented and deficient systems respectively is accounted for.

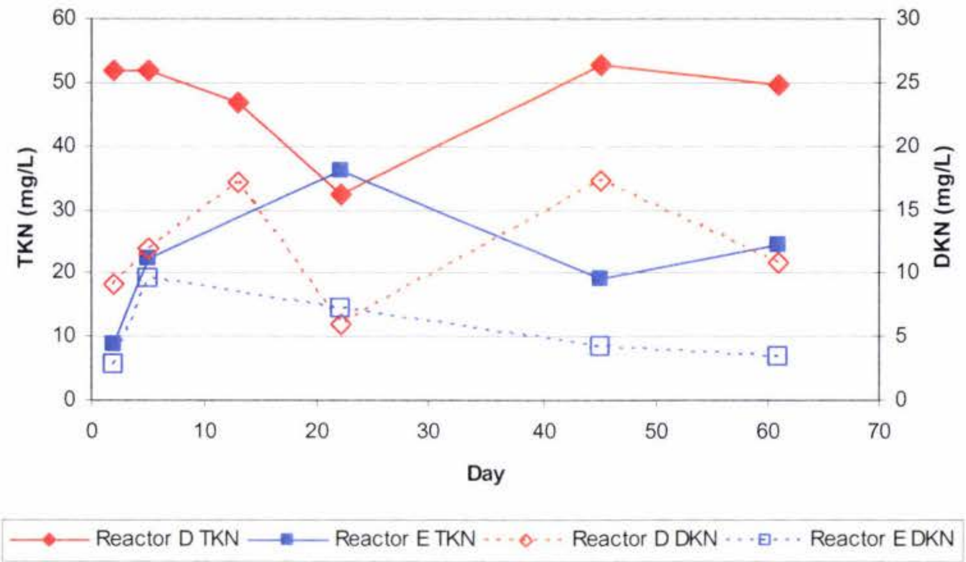


Figure 6.15 Total and Dissolved Kjeldahl Nitrogen Effluent Concentrations within Reactor D and Reactor E

Of more significance is the level of dissolved nitrogen within the effluents. Within the nitrogen deficient system (Reactor E) the DKN concentration in the effluent drops to 3 mg/L after 60 days, while the supplemented reactor has a concentration fluctuating between 10 and 18 mg/L.

The nitrogen balance for the nitrogen supplemented reactor (Table 6.5) shows that almost 20 % of the nitrogen entering the system (as soluble nitrogen) is not consumed or utilised and passes straight through the system. Interestingly, the amount of soluble nitrogen passing through the deficient system is 14 % of the influent, this shows that the nitrogen fixing bacteria produce a surplus of nitrogen.

Table 6.5 Nitrogen Balances for Reactor D and Reactor E (Day 61)

Reactor		In		Out		
		Feed (mgN/d)	N-Fixed (mgN/d)	N-soluble (mgN/d)	N-solid (mgN/d)	N-tot (mgN/d)
D	Nitrogen Supplemented	114.1	0.0	21.8	77.2	99.0
E	Nitrogen Deficient	2.2	47.1	7.0	42.3	49.3

6.9.4 Phosphorus Treatment

Phosphorus was added to both reactors at the same level of 10.5 mg/L total phosphorus (TP) and 5.5 mg/L dissolved (DRP).

The concentration of TP within the effluents of both reactors was found to be 8.9 mg/L and 10.9 mg/L for the nitrogen supplemented and deficient reactors respectively (Figure 6.16). The dissolved phosphorus however drops to 0.2 and 3.0 mg/L showing that although the total levels of phosphorus within the reactors is not decreasing significantly, the uptake of soluble phosphorus into micro organisms and its conversion to biomass can account for both the decrease in DRP and the maintenance of the TP.

At steady state the total phosphorus in both the feed and effluent should be equal with no accumulation within the reactor biomass. Lower concentrations within the effluent (during the initial stages) indicate that phosphorus is being accumulated within both systems.

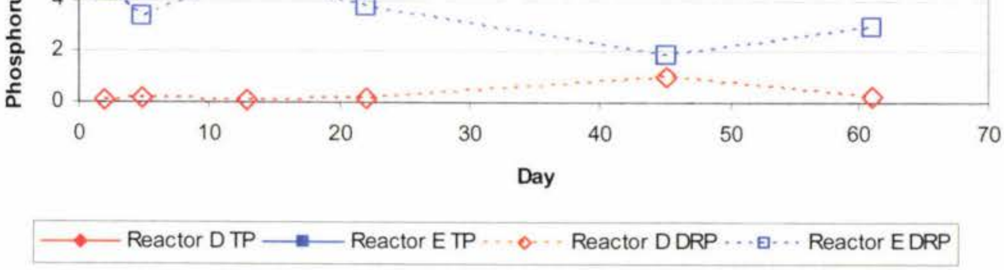


Figure 6.16 Total and Dissolved Phosphorus Effluent Concentrations within Reactor D (Nitrogen Supplemented) and Reactor E (Nitrogen Deficient)

6.10 Carbohydrate Production

The hypothesis stated that, in order to protect the nitrogen-fixing enzyme from high dissolved oxygen concentrations the micro organisms within Reactor E (nitrogen deficient) would generate increased amounts of extracellular polysaccharides (EPS).

Shown in Table 6.6 are the carbohydrate values found within Reactor D (nitrogen supplemented) and Reactor E (nitrogen deficient) on Day 80. Due to the higher biomass concentration within Reactor E the amount of carbohydrate present within the system is significantly higher than within Reactor D. On a specific basis however it was found that Reactor D had more carbohydrates present than Reactor E. The EPS calculation within the two systems shows that the expected high concentration within the nitrogen-deficient reactor was not present. Both these values are larger than the 3.1 mg Carbohydrate/g TSS found by Su and Yu (2005). The magnitude of difference between the literature and the results for Reactor D means that more investigation into the composition is required.

Table 6.6 Carbohydrate Concentrations in Reactor D and Reactor E taken on Day 80

	Equivalent Glucose Concentration (mg/L)		Carbohydrate Concentration (mg Glucose/g TSS)	
	Reactor D	Reactor E	Reactor D	Reactor E
Total	1368	2410	688	575
Soluble	14	31	7	7
Centrate	220	83	111	20
Solid	1354	2378	681	568
EPS	206	52	104	12

This result does not support the hypothesis that the operation of a nitrogen deficient granular reactor would result in a greater amount of EPS being generated than within a reactor supplemented with balanced levels of nitrogen. The reason behind this could be that the structure of a granule was enough to provide sufficient protection of the nitrogenase enzyme from oxygen stress without excess EPS production. This theory, however should be the subject of further research effort.

6.11 Long Term Viability

The viability of aerobic granules over a significant period of time has not thoroughly been investigated. The length of this study was only 75 days and this is a short length of time when compared to other studies such as McSwain *et al.* (2004), which went for 220 days, and Schwarzenbeck *et al.* (2004), which ran for 25 weeks.

6.12 Summary

This work has demonstrated, that for the first time, a successful adaptation of granular activated sludge to the treatment of nitrogen deficient feedstocks, through the acclimation of nitrogen fixing microorganisms. This is a novel and very important result, which could have implications for the treatment of many industrial wastewaters.

Two reactors were run: supplementation of nitrogen, in the form of NH_4Cl (Reactor D), and no supplementation of nitrogen (Reactor E) were used to determine whether granulation within a nitrogen deficient reactor was possible, and if so, what is the effect of nitrogen deficiency on a granular system.

Excellent granulation was generated in both the supplemented and deficient reactors, and biological nitrogen fixation was confirmed as an active process within the N-deficient reactor. The morphological and treatment characteristics found within the two reactors are shown in Table 6.7.

Table 6.7 Summary characteristics for the Reactor D (nitrogen supplemented) and Reactor E (nitrogen deficient) systems.

		Reactor D *Nitrogen Supplemented*	**Reactor E** *Nitrogen Deficient*
Image Analysis	Volume-based diameter (mm)	2.2	6.1
	Circularity	0.41	0.45
	Fractal Dimension	1.057	1.139
Individual Granules	Area (cm²)	0.03	0.39
	Equivalent Diameter (mm)	2.0	7.0
	Circularity	0.61	0.21
	Fractal Dimension	1.58	1.62
	Settling Velocity (cm/s)	0.88	1.42
	Specific Gravity	1.019	1.002
Mixed Liquor	Sludge Volume Index (mL/g)	125	48
	Suspended Solids (mg/L)	1550	3540
	EPS (mgGlucose/gTSS)	104	12
Effluent	Suspended Solids (mg/L)	290	160
	Soluble Carbon (mg/L)	17	14
	Carbon Treatment (%)	96.6%	97.4%
	TKN (mg/L)	50	24
	DKN (mg/L)	11	4
	Phosphorus (mg/L)	8.9	10.9

Microbial analysis using T-RFLP revealed that the bacterial population diversity was higher in the nitrogen deficient reactor.

The quality of effluent from each reactor was investigated. It was found that, over the course of the study, the nitrogen deficient reactor proved superior to its supplemented counterpart, particularly in nitrogen and solids discharge in the final effluent.

The granules generated under nitrogen deficient conditions were found to have significantly different properties to those generated in the supplemented reactor. Image analysis of the granular populations found that granules within the nitrogen deficient reactor were dramatically larger, less spherical and had an increased surface ruggedness (measured as the fractal dimension).

The need to protect the nitrogenase enzyme is the driving factor for the resulting granular macro structures and their surface characteristics. Nitrogen fixers are known for their highly filamentous nature, and this is clearly evident within the microscopy samples, and these filaments and large number of protrusions cause the increased fractal dimensions. The filaments themselves could also serve as structural support within the granule but also create voids within the structure.

Granules generated under nitrogen deficient conditions were better settling than those generated under supplementation, with faster settling times and lower sludge volume indices, despite having a lower density. This lower density could reflect voids or gel-formation within the granule structure. As a result of improved settling performance, the biomass concentrations within the nitrogen deficient reactor were significantly higher than those within the supplemented reactor.

The hypothesis of increased EPS production within the nitrogen deficient reactor, in order to protect the nitrogen fixation faculties, was not proven in this work, although the measured concentration was still found to be greater than reported values from literature (Su and Yu, 2005). This illustrates the potential that the structure of a granule may have provided sufficient alternative protection of the nitrogenase enzyme from oxygen stress, thus minimising any advantage in generation of large amounts of EPS. There is a clear requirement for further work in this area, should granulation of these systems be of continued interest.

This study has shown that, not only are granular nitrogen deficient reactors viable, but they also show significant advantages over supplemented reactors. However the viability of these granules over long periods has not been investigated, with typical granular system run for up to 220 days compared to the study's 75 days.

Chapter 7: Effect of Turbulence

7.1 Introduction

The general effect of turbulence and hydrodynamic shear forces on the formation and development of biofilms and granular systems has been studied. However, given the novelty of the process, the effect of turbulence on specific nitrogen fixing or nitrogen deficient systems has not been investigated.

In the case of biofilms it has been found that stronger hydrodynamic shear forces result in a much stronger biofilm, whereas if these forces are weaker then the structure of the biofilm is itself weaker and increasingly non-uniform and porous in nature (Kwok *et al.*, 1998; Chang *et al.*, 1991; Chen *et al.*, 1998; Rittmann 1982; van Loosdrecht *et al.*, 1995). The similarities between biofilm and granular systems suggest that these findings are analogous between biofilm and granular systems.

Research into aerobic granulation has found that under less turbulent, lower hydrodynamic shear conditions granulation did not occur and systems became dominated by fluffy flocculent biomass (Liu *et al.*, 2003b; Tay *et al.*, 2001; Tsuneda *et al.*, 2003; Yang *et al.*, 2003). These studies have found that a minimum specific upflow air velocity threshold is required before granules would be formed. Liu *et al.* (2003b) found that at an upflow velocity of 0.3 cm/s granules did not form and the system was dominated by 'conventional bioflocs'. In the same study, reactors run at an increased upflow velocity of 1.2 cm/s formed consistent, spherical granules. An early study by Beun *et al.* (1999) found that 'stable' granules did not form below 1.4 cm/s.

Typically, standard nitrogen fixing systems have been mixed using impellor technologies and sporadic aeration bursts to achieve the low oxygen concentration set points. Within aerobic granular systems, mixing is typically achieved using upflow aeration.

As this constant bubbling of air into the reactor results in a higher oxygen concentration than is typically found within nitrogen fixing systems it was hypothesised that for a granular nitrogen fixing system a balance must be met between increased hydrodynamic shear and increased oxygen concentration inhibiting the nitrogen fixation process.

7.2 The Reactors

The three reactors run, under nitrogen deficient conditions, during this experiment were Reactors E, F and G.

1. Reactor E: Tall reactor, large air pump, high oxygen concentration and high aeration rate [High turbulence, upflow air velocity of 1.2 cm/s].

2. Reactor F: Tall reactor, large air pump, high oxygen concentration and variable aeration rate [Moderate turbulence, upflow air velocity of 0.5 cm/s].The aeration rate was determined by the DO concentration; at low DO the aeration rate was at its maximum but as the DO increased the aeration rate decreased proportionately.

3. Reactor G: Tall reactor, small air pump, low oxygen concentration, on/off control and mixing using a flea and stirrer plate [Low turbulence, upflow air velocity less than 0.5 cm/s].

These three reactors were all run at 1 minute settling time to help select for larger particles (based on the finding presented in Chapter 5) and at an 8 hour total cycle time. The three Reactors were run for 60 days (90 hydraulic retention times) and monitored over this period.

It was expected that granules would form within Reactor E and Reactor F. However due to lack of turbulent conditions granule growth within Reactor G was not predicted.

7.3 General Observations

Growth within all 3 reactors occurred within a few days of start-up. Within the two turbulent Reactors E and F this growth occurred as the formation of granular bodies. Within the less turbulent Reactor G granular growth did not occur. Instead of granular bodies forming within the reactor, a sparse and thin biofilm formed on the surfaces within the reactor coating the probes and walls within.

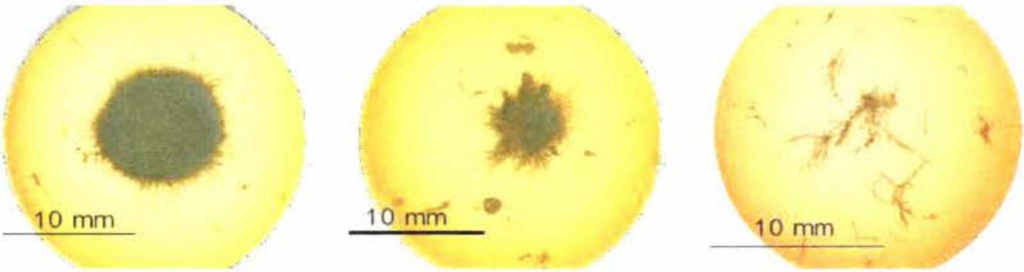


Figure 7.1 Individual granule comparisons at Day 60. Left granule from Reactor E (High Turbulence), middle granule from Reactor F (Moderate Turbulence), right granule from Reactor G (Low Turbulence).

Figure 7.1 shows typical samples taken from each reactor investigated within this study. At a high turbulence of 1.2 cm/s distinct spherical entities were observed. Similar findings were reported by Beun *et al.* (1999) and Liu et al., (2003b). And at moderate turbulence of 0.5 cm/s granular shapes were still observed. This agrees with the results from Liu *et al.* (2003b) which set the minimum superficial upflow air velocity threshold for granule formation between 0.3 and 1.2 cm/s.

From the images shown Figure 7.1 it is evident that for nitrogen-fixing systems granules grown at an upflow air velocity of 1.2 cm/s (Reactor E) have significantly less filamentous outgrowths and are optically denser than those grown at 0.5 cm/s (Reactor G) as well as the biomass generated in the low turbulence reactor. The interesting morphological appearance of these granules could be attributed to the relatively low carbon loading within the feed. Most studies find that low organic loading rates, such as the one used in this study, resulted in granules that were more irregular and fluffy (Liu *et al.*, 2005; Moy *et al.*, 2002; Tay *et al.*, 2004a & 2004b). Another cause for surface features could be the form of carbon substrate; Tay *et al.* (2001) found that reactors fed on different substrates resulted in significantly different granule structures.

By convention, it was expected that the lack of granulation in Reactor G was caused by the lack of mixing and low upflow air velocity. Due to the sporadic aeration regime, mixing akin to that occurring within the other two reactors was not provided. The implementation of stirrer was an attempt to counter this but due to reactor size and design the mixing occurred only within the lower 100 to 150 mm of the reactor and was insufficient within the 1 m tall reactor. The reactor was continued to be run in an attempt to achieve granulation. This attempt however was unsuccessful.

The stirrer was able to dislodge some of the biofilm growth from the walls of the reactor. This biomass was highly filamentous and semi-translucent. An example of this biomass was shown in Figure 7.1. Distinguishing individual particles was difficult due to the entwining nature of the filaments. This convolution means that image analysis of a large number of individual particles was impractical and unable to deliver a proper representation of the system population.

As a consequence of this failure to granulate, results from this reactor were incomparable to the other two reactors run within this study and results have been omitted from the following analyses.

These findings agree with those found in literature (Beun *et al.*, 1999; Liu *et al.*, 2003b; Tay *et al.*, 2001; Tsuneda *et al.*, 2003; Yang *et al.*, 2003): low turbulence regimes do not have sufficient hydrodynamic forces to facilitate granulation of biomass within the reactor.

7.4 Population Characterisation

T-RFLP investigations found that the microbial populations within both the high turbulence (Reactor E) and moderate turbulence (Reactor F) systems were similar (Figure 7.2). This shows that differences in their structural characteristics are not due to variation in the make-up of the bacterial populations.

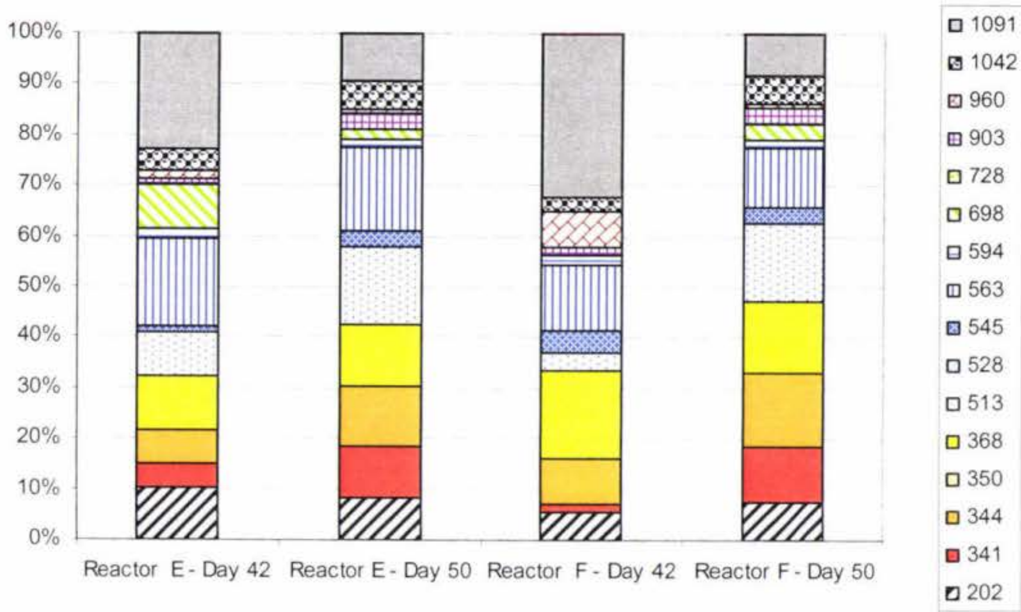


Figure 7.2 T-RFLP Population study. Bands identify different terminal fragments that determine population fractions within the reactors (as denoted in the legend).

7.5 Nitrogen Fixation

The test for nitrogen fixation was carried out, Figure 7.3. This found that despite the higher driving force for permeation of oxygen into the granules within the high turbulence system (Reactor E), fixation still occurs. This is confirmed by the acetylene reduction assay results. The clear presence of ethylene peaks shows that the reduction of acetylene to ethylene did occur.

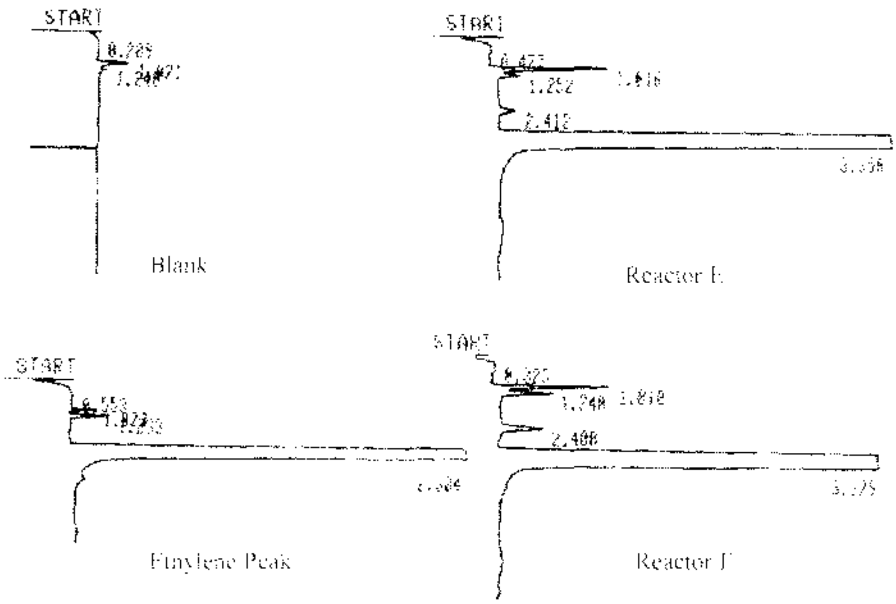


Figure 7.3 Acetylene Reduction Chromatograms. The ethylene standard shows the peak for ethylene (product of acetylene reduction) and this peak is found in both results from Reactor E and Reactor F.

7.6 Morphology

Shown in Figure 7.4 are mixed liquor images from Reactor E (High Turbulence) and Reactor F (Moderate Turbulence). Granules within Reactor E are better formed than those within the moderate turbulence Reactor F. Granules within Reactor F appear to be fragmented and ill-formed while those granules within Reactor E have that spherical core surrounded by filamentous off-shoots.

Figure 7.4: Mixed Liquors. Left Reactor E (High Turbulence), right Reactor F (Moderate Turbulence) (Taken Day 72)

7.6.1 Image Analysis: Granular System

Analyses were carried out on data collected on Day 61 of the study. Investigation into the size of granules within the two reactors containing granules found that both reactors had similarly sized populations with mean volume based cumulative distribution values of 6.1 and 6.9 mm diameters for the high turbulence (Reactor E) and moderate turbulence (Reactor F) systems respectively. Shown in Figure 7.5 is this size distribution found on Day 61. Both reactors maintained these size distributions for over 50 HRT despite changes in some other characteristics. This shows that reactors were capable of sustaining a granular population at within nitrogen fixing reactors with superficial upflow air velocities of 1.2 cm/s and 0.5 cm/s over a period of several months; longer term studies are required to determine the stability of granules in nitrogen deficient systems over longer time periods.

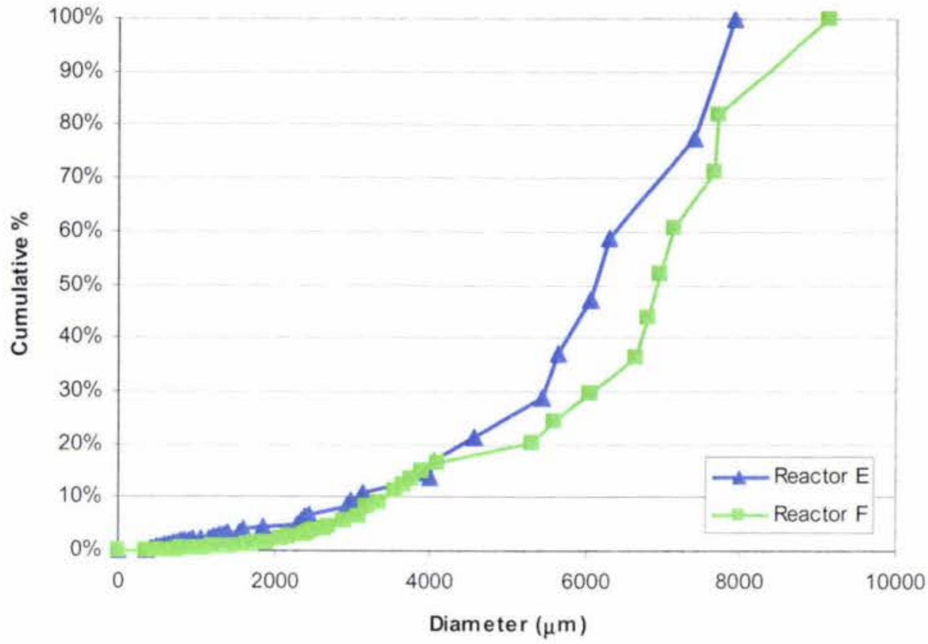


Figure 7.5 Volume-based cumulative distribution values within Reactor E and Reactor F at steady state, on Day 61.

It was found that the edges of granules within the high turbulence reactor had a slightly more circular shape (Table 7.1) than those that were generated within the moderate turbulence reactor. This irregularity was further confirmed through the Fractal Dimension calculated using the Zartarian *et al.* (1997) method. These two numbers show that the Reactor E with its high level of turbulence was capable of generating granules that were increasingly circular and the 'rugged' or filamentous nature of the granule edge was less than in the Reactor F with its moderate turbulence. Tay *et al.*(2001a) found similar results win increased turbulence increasing the smoothness of granules.

Table 7.1 Edge characteristics of granules within Reactor E (High turbulence) and Reactor F (Low turbulence) taken on Day 61.

	Reactor E	Reactor F
Circularity	0.451	0.341
Fractal Dimension	1.139	1.202

Other studies have found that as granules mature their surfaces become increasingly smooth and regular (Beun *et al.*, 2002; Jang *et al.*, 2003). In comparison to these studies, the length of this study into nitrogen-fixing granules is significantly shorter and opens the possibility that the 'final' form of granules generated could be significantly smoother and more regular.

Shown in Table 7.1 is the mean circularity of granules on Day 61. The circularity within high upflow air velocity Reactor E remained constant throughout the end stages of the experiment. Granules within Reactor F became increasingly more irregular and this is reflected by the relatively reduced circularity.

7.6.2 Image Analysis: Individual Granules

On Day 72 fifteen individual granules were taken for analysis. Shown in Table 7.2 are the granule characteristics measured within both the high turbulence (Reactor E) and moderate turbulence (Reactor F) systems. These values were calculated without weighting granules based on their volume, and consequentially, the equivalent average diameter of granules within Reactor E was found to be higher than within Reactor F.

The individual granules in both reactors were found to have a low circularity value. But the difference between the two systems was still quite obvious: those found within Reactor E were found to have almost twice the circularity value of those found within Reactor F (0.21 compared to 0.10 respectively).

The equivalent circular diameter of granules is larger within Reactor E, 7.0 mm, than Reactor F, 5.2 mm, and when this is compared to the Feret's Diameter the potentially elongated and random shapes associated with Reactor F are evident.

Table 7.2 Reactor E (High Turbulence) and Reactor F (Moderate Turbulence) Granule Characteristics

	Reactor E		Reactor F	
Characteristic	**mean**	***std dev.***	**mean**	***std dev.***
Area (cm^2)	0.39	*0.12*	0.22	*0.08*
Perimeter (cm)	4.97	*1.00*	5.43	*1.59*
Circularity	0.21	*0.08*	0.10	*0.03*
Ferets Diameter (mm)	8.29	*1.32*	7.22	*1.52*
Equivalent Diameter (mm)	7.00	*1.05*	5.17	*0.90*
Fractal Dimension	1.62	*0.03*	1.71	*0.03*
Settling Velocity (cm/s)	1.42	*0.23*	0.89	*0.16*
Density (kg/m^3)	1002	*1*	1001	*1*

7.7 Granule Settleability

The buoyancy density of granules within the two reactors shows the similarity between them. Despite this the settling velocity of granules within Reactor E was significantly higher (1.42 cm/s) than that of granules within Reactor F (0.89 cm/s). This settling velocity for the moderate turbulence Reactor F is similar to those values for acetate fed reactors that are found within the literature (*see Literature Review Chapter 2 Table 2.7*). However, the settling velocities found within the high turbulence reactor are higher than those values found for reactors fed with the same carbon source. Within studies using alternate carbon sources this settling velocity is comparable.

The packing of solids within the reactor can be described by the sludge volume index (SVI) this is shown in Figure 7.6. This shows that Reactor E, run under higher turbulence levels, was found to have comparable (but slightly better) solid settleability with Reactor F. Further comparisons found that the final SVI value of near 50 mL/g for both reactors show similarities with results from literature (Jang *et al.*, 2003; McSwain *et al.*, 2004; Moy *et* al., 2002; Su an Yu, 2005; Wang *et al.*, 2004) and was dramatically better than non-granulating sludge generated under similar nitrogen deficient and SBR conditions (Dennis *et al.*, 2004; Sheker *et al.*, 1993).

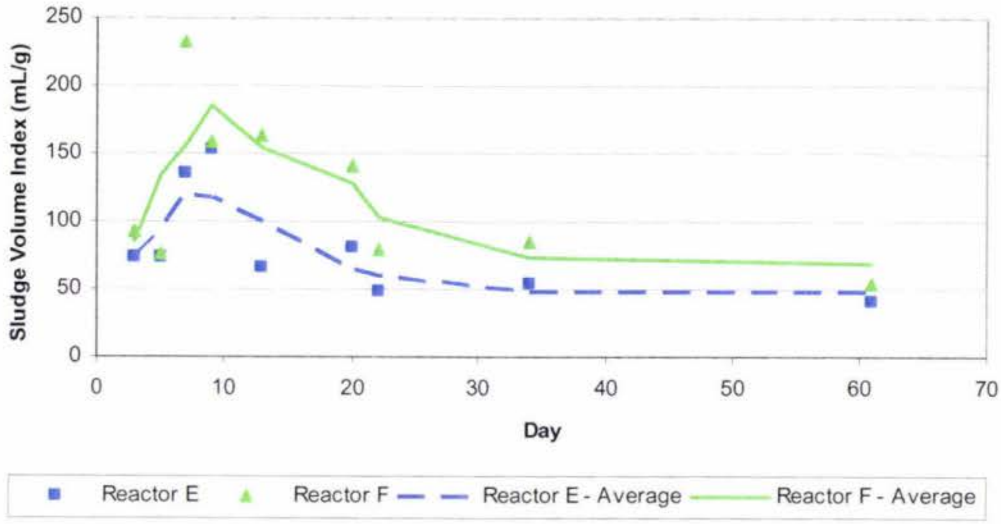


Figure 7.6 Sludge Volume Index of solids found within Reactor E (High Turbulence) and Reactor F (Moderate Turbulence)

7.8 Effect of Turbulence on Nitrogen-Fixing Granular Structures

A correlation to determine the effect of the turbulence variable within the two reactors was carried out and is shown in Table 7.3. The major outcomes from this analysis were that increasing the turbulence within the system resulted in granules with a more consistent edge characteristic as the fractal dimension drops to a value representing a less rugged edge and the granule becomes more circular. The other noticeable effect is the increased settling velocity at higher aeration levels. Increased settling velocity is important within granular systems as it allows retention of biomass despite the restrictions of shortened settling times. This shows that in this study, running reactors under more turbulent conditions results in more desirable, faster settling, granular structures.

Table 7.3 Correlation for Reactor E (High Turbulence) and Reactor F (Moderate Turbulence)

	Reactor	*Turbulence*	*Density*	*Fd*	*Area*	*Perim.*	*Circ.*	*Feret*	*S. Velocity*
Reactor	1.00								
Turbulence	-1.00	1.00							
Density	-0.48	0.48	1.00						
Fd	0.85	-0.85	-0.47	1.00					
Area	-0.68	0.68	0.19	-0.61	1.00				
Perim.	0.18	-0.18	-0.27	0.28	0.36	1.00			
Circ.	-0.70	0.70	0.24	-0.75	0.40	-0.56	1.00		
Feret	-0.36	0.36	-0.09	-0.28	0.86	0.66	0.13	1.00	
S. Velocity	-0.82	0.82	0.44	-0.76	0.79	0.00	0.58	0.57	1.00

7.9 Treatment Performance

A comparison of the treatment efficiencies of the two granular reactors was carried out to determine the carbon removal occurring and also the levels of solids, nitrogen and phosphorus in the effluent.

7.9.1 Reactor Solids Level

The total suspended solids values measured during this experiment are shown in Figure 7.7. Initially the focus of this experiment was on the start-up stages of the granulation process; however it became apparent during subsequent analysis and literature investigation that the start-up of a granular system takes a significantly longer period than was first assumed. Following a rapid increase in the solids level within both reactors washout occurred and the solids level dropped. After the populations stabilised they began to increase and by Day 60 they had reached biomass concentrations of 4.0 g/L within the Reactor E (high turbulence), and 5.0 g/L within Reactor F (moderate turbulence). Jang *et al.* (2003), with a slightly lower organic loading rate of 2.5 kgCOD/(m^3.d), reported a similar solids concentration of 5.8 g/L 60 days after reactor start-up whilst McSwain *et al.* (2004) and Tay *et al.* (2001), with organic loading rates of 2.4 and 6.0 kgCOD/(m^3.d) respectively, found the solids level within systems to increase above 8.5 g/L. Some of this could be attributed to the difference in settling velocity with more turbulent systems requiring more time to become quiescent and allowance of particles to begin settling.

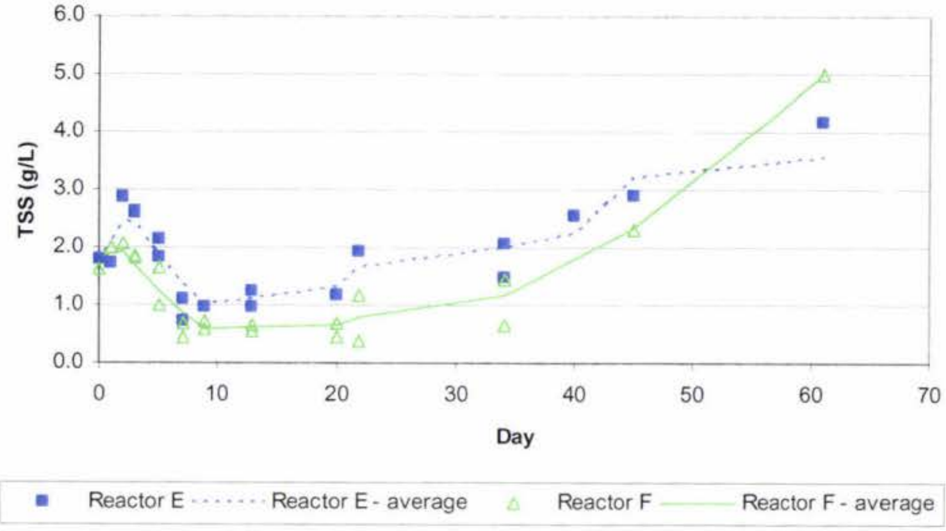


Figure 7.7 Total mixed liquor suspended solids found within Reactor E (High Turbulence) and Reactor F (Moderate Turbulence).

Figure 7.8 shows the effluent discharge occurring within each reactor is at a similar level of around 0.20 g/L for the first month. This consistent level of solids washout within the effluent while the level of solids within the mixed liquor increases implies that a similar fraction of the reactor mixed liquor is being washed out. At Day 60 the solids level within the effluent of the high turbulence Reactor E was found to have decreased slightly to 0.15 g/L but within the reactor with only moderate turbulence Reactor F the washout had decreased to a level just greater than 0.05 g/L.

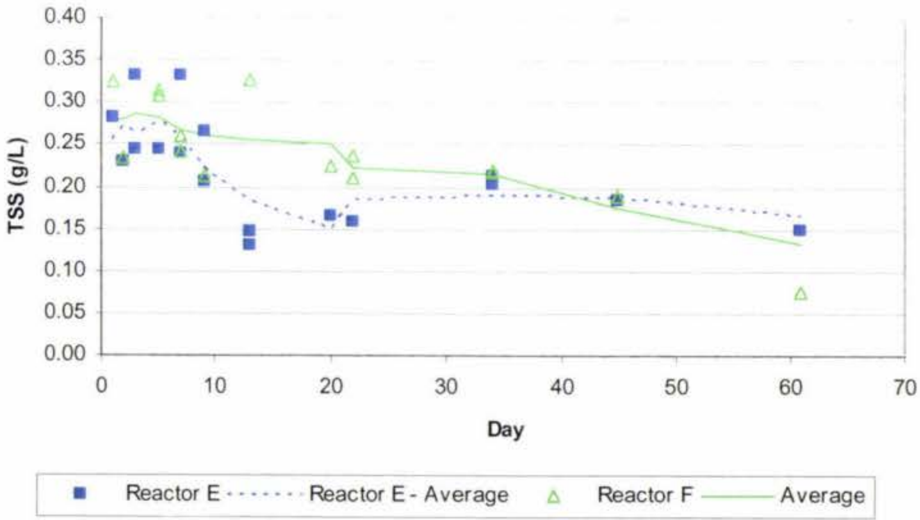


Figure 7.8 Total effluent suspended solids found within Reactor E (High Turbulence) and Reactor F (Moderate Turbulence)

Increased levels of turbulence were expected to result in increased levels of washout due to increased shear causing higher levels of surface attrition. However, this was not found to occur until towards the final 15 days of the study where retention of biomass within the moderate turbulence system (Reactor F) increased significantly, dropping from an effluent solids concentration of 220 to140 mg/L while the mixed liquor solids concentration doubled from 2.2 to 5.0 g/L.

Within the low turbulence Reactor G, effluent suspended solids slowly built up to reach a final mixed liquor concentration of only 0.4 g/L suspended solids, with an effluent suspended solids concentration of 0.20 g/L on Day 63, revealing the lack of growth that was occurring.

7.9.2 Carbon Removal

The influent total organic carbon concentration of the feed was 500 mg/L in the form of sodium acetate. Both reactors were found to be capable of removing a significant percentage of influent carbon (utilised for respiration and growth). The variation in the treatment efficiency over time is shown in Figure 7.9 and shows that the high turbulence reactor (E) had an average treatment efficiency of 97.5 % while the moderate turbulence in Reactor F resulted in a slightly lower efficiency of 96.5 %.

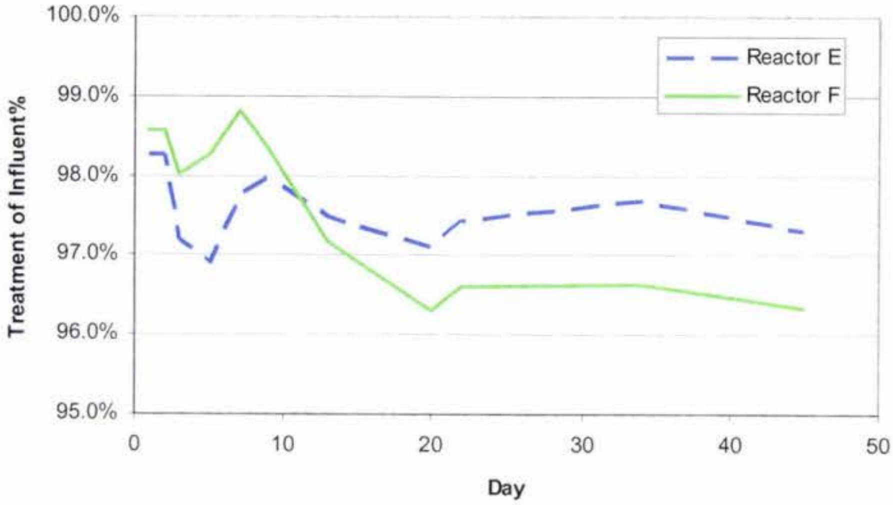


Figure 7.9 Treatment efficiencies within the Reactor E (High Turbulence) and Reactor F (Moderate Turbulence).

The dissolved oxygen concentration within each reactor was measured. Due to the minimal oxygen utilisation rates within the low turbulence reactor, changes were only seen during decant and feed cycles followed by a gradual regeneration to saturation oxygen concentration.

Shown in Figure 7.10 are the dissolved oxygen (DO) profiles within both the high turbulence (Reactor E) and moderate turbulence (Reactor F) systems. This shows the DO recovery curves, where the oxygen concentration drops as the substrate is consumed and then returns to saturation over time. The length of the drop indicates that both reactors consumed substrate over a similar period of time (between 1.5 – 2.0 h). The magnitude of the drop of DO within the two systems can be explained through mass transfer and replenishment rates. With higher turbulence and thus mass transfer within Reactor E the drop is much smaller when compared to the moderate turbulence Reactor F.

This shows that the dissolved oxygen concentration was not providing a significant rate limitation for the nitrogen-fixing systems under these conditions. With the treatment levels remaining at high efficiencies and solids levels at comparable levels to other studies, growth within these nitrogen fixing granular systems has not been shown to be inhibited by the elevated dissolved oxygen concentrations. This phenomenon may reflect a highly advantageous aspect of granules, namely that of providing microniches where particular bacterial mechanisms (like nitrogen fixation under low oxygen environments) can flourish, to the benefit of the overall system.

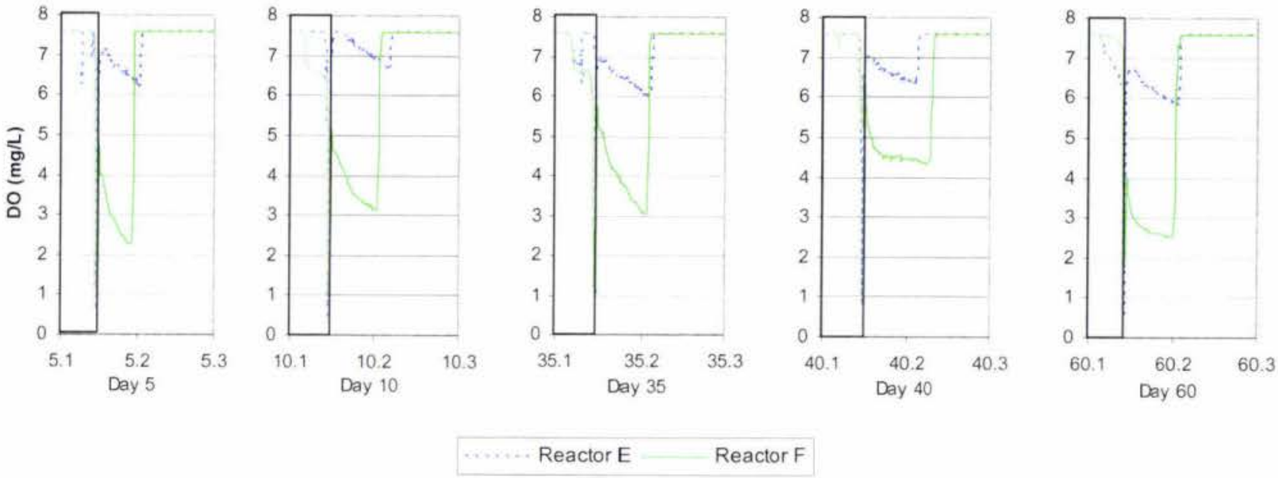


Figure 7.10 Dissolved Oxygen Profiles within the Reactor E (High Turbulence) and Reactor F (Medium Turbulence). The shaded areas denote time decant and feed periods.

7.9.3 Nitrogen Treatment

The nitrogen, in the forms of Kjeldahl, total (TKN) and dissolved (DKN), nitrogen oxides (NO_x) and ammonium (NH_4^+) was measured in the feed and effluent streams from both reactors. Shown below in Table 7.4 is the nitrogen concentration found within the influent.

Table 7.4 Influent Nitrogen compounds within Reactor E (High) and Reactor F (Moderate)

Component	Reactor E (mg/L)	Reactor F (mg/L)
NO_x	0.03	0.03
TKN	6.8	4.3
DKN	4.8	3.2

Kjeldahl nitrogen concentrations are shown in Figure 7.11. The Kjeldahl nitrogen concentration within both reactors shows a significant increase over that which enters via the feed, again reflecting the action of the bacteria carrying out fixation of nitrogen into both reactors. The increase in the effluent TKN can be directly attributed to the amount of biomass being washed out from both of the reactors, while the DKN in and out of the system did not change significantly, and remained consistently low, re-emphasising the excellent nutrient discharge control which can be exerted by a nitrogen fixing system.

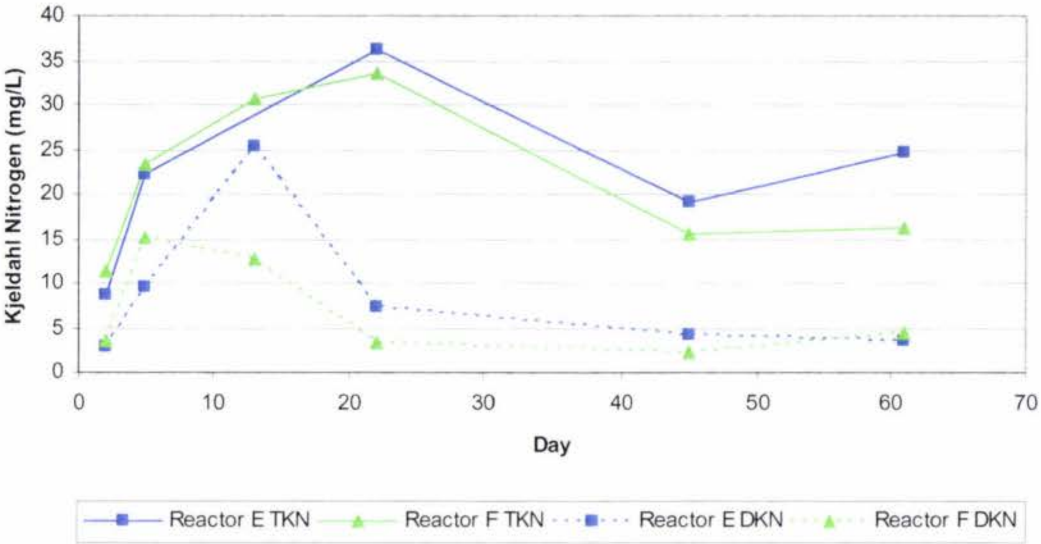


Figure 7.11 Total and Dissolved Kjeldahl Nitrogen Effluent Concentrations within Reactor E (High) and Reactor F (Moderate)

Nitrogen balances (Table 7.5) reveal that the amount of nitrogen fixed within the high turbulence system is over 50 % more than the amount fixed within the moderate turbulence reactor. This is interesting as the assumption was that higher oxygen concentrations, as a consequence of the high turbulence, would result in lower levels of fixation. The ratio of soluble to solid nitrogen being discharged, 1:6.0 mgN/d and 1:2.69 mgN/d for the high and moderate reactors respectively, shows that a high proportion of the soluble nitrogen that was fixed is being discharged with utilisation by the microbes within the system.

Table 7.5 Nitrogen Balances for Reactor E and Reactor F (Day 61)

Reactor		In		Out		
		Feed (mgN/d)	N-Fixed (mgN/d)	N-soluble (mgN/d)	N-solid (mgN/d)	N-tot (mgN/d)
E	High Turbulence	2.2	47.1	7.0	42.3	49.3
F	Moderate Turbulence	2.2	30.3	8.8	23.7	32.5

7.9.4 Phosphorus Treatment

Phosphorus was added to both reactors at the same level of 10.5 mg/L total phosphorus (TP) and 5.5 mg/L dissolved (DRP).

The concentration of TP within the effluents of both reactors was found to be 10.9 mg/L and 7.6 mg/L for the high turbulence and moderate turbulence reactors respectively (Figure 7.12). The lower value within the moderate turbulence value can be attributed to an increased retention of biomass within the system, as the mixed liquor suspended solids concentration continued to increase throughout the study period, while the level of solids discharged in the effluent continued to decrease.

Both reactors show similar utilisation levels of dissolved phosphorus with drops of 3.0 and 2.6 mg/L being found in the two systems. This shows that the effect of turbulence does not directly affect the uptake of phosphorus.

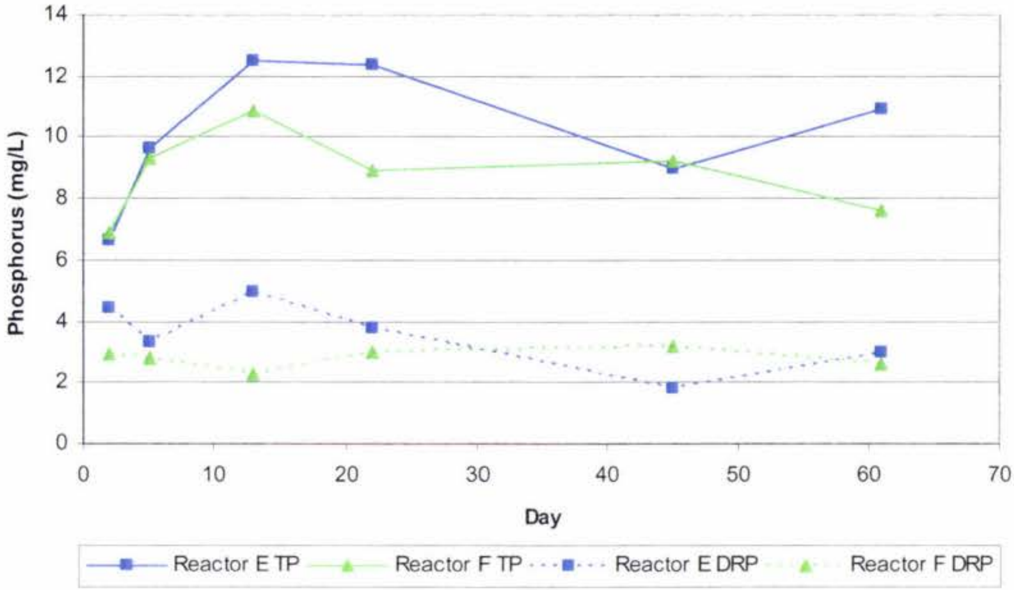


Figure 7.12 Total and Dissolved Phosphorus Effluent Concentrations within Reactor E (High Turbulence) and Reactor F (Moderate Turbulence)

7.10 Carbohydrate Production

It has been found that increased levels of turbulence and hydrodynamic shear result in the overproduction of extracellular carbohydrates (Chen *et al.*, 1998; Ohashi and Harada, 1994; Trinet *et al.*, 1991). These carbohydrates can be separated into the major subcategories of polysaccharides and proteins. The ratio between these two categories was found to vary from 5:1 to 15:1 (polysaccharides:proteins) increasing with increased turbulence (Tay *et al.*, 2001). The amount of extracellular polysaccharide (EPS) is often regarded as having significant effect on reactor characteristics. Increased levels of EPS increase the way that cells bind together as well as the integrity of any matrix the cells are part of (Tsuneda *et al.*, 2001) while the disappearance of aerobic granules has been found to coincide with a drop in the EPS concentration (Tay *et al.*, 2001).

Carbohydrate EPS concentrations were measured within both the high and moderate turbulence reactors. Both reactors, having demonstrated similar suspended solids values, with high turbulence reactor having a slightly higher TSS value, were found to have similar concentrations of soluble and centrate EPS concentrations. This led to a very similar EPS reading of 12 and 11 mg Glucose equivalents/g TSS for the high and moderate turbulence reactors respectively (Table 7.6). Comparison to literature however finds that these values are larger than the 3.1 mg Carbohydrate/g TSS found by Su and Yu (2005).

The total carbohydrate calculation within the high turbulence, Reactor E, was significantly higher than within the moderate Reactor F, this is interesting as the solids levels within the two reactors are very similar at this point. Turbulence was found not to have an influence on the amount of EPS within the systems.

Table 7.6 Carbohydrate-based EPS concentrations within Reactor E (High) and Reactor F (Moderate) taken on Day 80

	Equivalent Glucose Concentration (mg/L)		**Carbohydrate Concentration (mg Glucose/g TSS)**	
	Reactor E	**Reactor F**	**Reactor E**	**Reactor F**
Total	2410	1616	575	324
Soluble	31	26	7	5
Centrate	83	79	20	16
Solid	2378	1590	568	319
EPS	52	53	12	11

7.11 Summary

Three reactors were run under varying levels of turbulence. Two of these systems, Reactors E and F, were run under high (1.2 cm/s upflow air velocity) and moderate (0.5 cm/s) turbulence conditions respectively and formed granules with a median size of 6.1 and 6.9 mm for each system. However, the third system, Reactor G, run at a low turbulence (below 0.5 cm/s), was incapable of granulation. This confirmed that a minimum turbulence within a system is required in order for granulation to occur. Shown in Table 7.7 are the summary characteristics for the Reactor E and Reactor F.

Table 7.7 Summary Characteristics for the granulating systems Reactor E (high turbulence) and Reactor F (moderate turbulence)

		Reactor E *High Turbulence*	**Reactor F** *Moderate Turbulence*
Image Analysis	Volume-based diameter (mm)	6.1	6.9
	Circularity	0.45	0.34
	Fractal Dimension	1.139	1.202
Individual Granules	Area (cm^2)	0.39	0.22
	Equivalent Diameter (mm)	7.0	5.2
	Circularity	0.21	0.1
	Fractal Dimension	1.62	1.71
	Settling Velocity (cm/s)	1.42	0.89
	Specific Gravity	1.002	1001
Mixed Liquor	Sludge Volume Index (mL/g)	48	70
	Suspended Solids (mg/L)	3540	4990
	EPS (mgGlucose/gTSS)	12	11
Effluent	Suspended Solids (mg/L)	170	130
	Soluble Carbon (mg/L)	14	18
	Carbon Treatment (%)	97.4%	96.3%
	TKN (mg/L)	24	16
	DKN (mg/L)	4	4
	Phosphorus (mg/L)	10.9	7.6

The granules generated at high turbulence (Reactor E) were found to be more circular and have a less rugged edge characteristic than those generated within the moderate turbulence system (Reactor F).

The high turbulence granules were faster settling than those generated under moderate turbulence and had a lower sludge volume index despite granules generated under both sets of conditions having similar densities.

A higher mixed liquor solids content was found within the moderate turbulence reactor, and the level of solids within the effluent was lower than within the higher turbulence reactor. The carbon removal efficiency were similar within both systems and reflected an excellent level of organic carbon removal.

The hypothesis that a certain turbulence threshold must be achieved in order for granulation to occur was confirmed, with granulation not occurring when the superficial upflow air velocity of the reactor was below 0.5 cm/s. When the upflow air velocity was maintained at 0.5 cm/s the oxygen uptake rate was greater than at a higher air velocity of 1.2 cm/s. This shows that a high dissolved oxygen concentration can be overcome. This is most likely through the effect of mass transfer limitation caused by granule structure and EPS production.

Chapter 8: Conclusions and Recommendations

This Masters thesis details the successful development of an aerobic granular system capable of treating nitrogen deficient wastewater through nitrogen fixation. This is a novel and very significant result, and could have implications for the treatment of many industrial wastewaters.

Nitrogen fixation and granulation are two unique technologies that have been separately utilised in the treatment of industrial wastewaters. However, systems uniting the principles of these two treatment processes have not been investigated.

Nitrogen fixation allows treatment of wastewaters where the amount of available nitrogen is a limiting factor. However, these systems are often plagued with poor sludge settleability and, due to the sensitivity of the enzyme responsible for fixation, must operate at low levels of oxygen concentration.

Conversely aerobic granular systems have been found to operate with good sludge settleability and, due to the conventional method of mixing and turbulence, at high levels of oxygen concentration. Previous studies have found that there is a turbulence threshold below which granules will not form. This led to the potential conflict within a system that utilised both of these technologies: for nitrogen fixation to occur a low oxygen concentration was required, while for granules to form the high level of turbulence meant a high oxygen concentration was required.

Using a specially developed image analysis technique, characteristics of granules were investigated in order to compare the effects of operating conditions on the shape, size and morphology of granules. Other analyses were also utilised in order to understand the biochemical responses to varying operating conditions.

The investigation into the effect of settling time on the granule formation process found that the time allowed for a reactor to settle did have an effect on the formation characteristics of granules, but not whether granules would be formed within the reactors. Three reactors were run at 1, 2 and 15 minutes settling time. It was found that the type of granules grown in the 1-minute were significantly different from the type of granules grown in both the longer settling reactors. Granules grown under the 1-minute settling time reactor were larger than those within the 2 and 15-minute settling time reactors and had a more irregular edge characteristic while both the 2 and 15-minute systems had similar values. This shows that at some point between 1 and 2 minutes settling is a critical point where the characteristics of granules change. This also shows the importance of reactor washout for the determination of granule size.

The investigation into the effect that nitrogen deficiency had on granulation found that it was possible to generate granules in nitrogen deficient treatment systems. Certain types of bacteria have an enzyme, nitrogenase, capable of fixing atmospheric nitrogen and converting it into an available form for the bacteria to use. Fixation was found to occur in systems subjected to nitrogen deficient conditions, and as the amount of nitrogen added to a system was increased, nitrogen fixation ceased. The physical differences between the granules grown under nitrogen-rich and nitrogen-deficient conditions was evident in the difference in the granule size and structure. Those granules grown without nitrogen supplementation were denser and larger than those grown with. The increase in size resulted in limited penetration of oxygen into the granule. Zones of low oxygen concentration were created within a granule that would allow bacteria to fix nitrogen.

The investigation into the effect of turbulence on the formation of granules has previously been documented. Mixing, and turbulence, within these reactors is generated through continuous aeration using compressed air. This constant aeration however means that the oxygen concentration within the reactor is maintained at high levels. This interferes with the some microbial processes, most notably nitrogen-fixation, but has been found to be a requirement for generation of granules. However as no literature has documented the effect of turbulence on nitrogen deficient systems this investigation endeavoured to determine the influence this had on granular characteristics. A reactor run under highly turbulent aeration conditions was compared with a reactor run under a variable aeration level that had a minimum baseline level of aeration and a reactor run without this minimum threshold. It was found that without a minimum turbulence threshold granules were not generated and a biofilm became the dominant microbiological form within the reactor. The comparison of high and moderate aeration reactors found that granules grown under the less turbulent regimes were more filamentous, irregular and had slower settling velocities than those grown under greater turbulence.

Comparison of carbon treatment found that both nitrogen deficient and supplemented systems were capable of similar levels of removal with typical removal efficiencies greater than 95 % for both sets of systems.

The levels of total Kjeldahl nitrogen within the effluent of the nitrogen deficient system was found to be half that found within the supplemented system showing clearly that it has significant advantages when it comes to effluent quality.

The salient discoveries within this study were that:

1. It was possible to generate aerobic granules using nitrogen deficient wastewater.
2. Decreased settling time was found to result in larger denser granules.
3. A minimum turbulence was required in order for granules to form.
4. Carbon removal levels are not impacted when supplemented systems are compared to deficient systems; while nitrogen levels are significantly lower for systems where fixation is the source of nitrogen for growth.
5. Nitrogen granules are an interesting and significant discovery with the potential for further investigation in future investigations.

The scope of future work on aerobic nitrogen-fixing granular technology based on this study has the potential to expand down a number of different avenues. The most significant area of investigation would be the long term viability of granules developed using this technology, with other granular studies running for much longer periods of time.

Further development of the image analysis techniques, including the distributions of granules within reactors and their surface characteristics would allow for a greater understanding of the influence that the key parameters such as settling time and turbulence have on microbial development.

Research to acquire a greater knowledge of the microbial characteristics of granules grown within aerobic, nitrogen deficient systems, would provide a significant insight into the structure, formation and distribution of different microbial populations within a granule. Understanding of the nutrient, carbon and oxygen levels, within specific regions of a granule and how these components are transported internally within granules would also help facilitate development of systems.

Ultimately, optimisation of nitrogen-fixing granular systems using synthetic wastewaters would ideally lead to the development of granular systems capable of treating real wastewaters that are deficient in nitrogen. This could lead to significant commercial and industrial success in the form of a cheaper, high performance, treatment system.

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