İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

GROWTH AND STORAGE KINETICS OF ACTIVATED SLUDGE SYSTEMS OPERATED WITH VARIOUS CARBON SOURCES FOR DIFFERENT SLUDGE AGES

Ph.D. Thesis by
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Department : Environmental Engineering

Programme : Environmental Biotechnology

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DEĞİŞİK KARBON KAYNAKLARINDA ÇALIŞTIRILAN AKTİF ÇAMUR SİSTEMLERİNDE FARKLI ÇAMUR YAŞLARINDA ÇOĞALMA VE DEPOLAMA KİNETİĞİ

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I would like to dedicate this work to its real owner; Ülkü AKTAŞ ÇIĞGIN.

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ABBREVIATIONS

Acetyl-CoA : Acetyl-Coenzyme A
AMP : Adenosine Monophosphate
ARP : Available Reaction Potential
ASM1 : Activated Sludge Model No. 1
ASM3 : Activated Sludge Model No. 3
ATP : Adenosine Triphosphate
$\mathbf{b_H}$: Endogenous Respiration Rate of Heterotrophs [T^{-1}]
$\mathbf{b_{STO}}$: Endogenous Respiration Rate of Storage Products [T^{-1}]
C:N : Carbon to Nitrogen Ratio
CoA : Free Coenzyme A
COD : Chemical Oxygen Demand
CSTR : Continuously Stirred Reactor
DO : Dissolved Oxygen
FISH : Fluorescence in Situ Hybridization
$\mathbf{f_{ES}}$: Fraction of Soluble Metabolic Products
$\mathbf{f_{EX}}$: Fraction of Particulate Metabolic Products
$\mathbf{f_P}$: Fraction of Particulate Inert COD Generated in Biomass Decay
G6P : Glucose-6-Phosphate
GRH : Growth Rate Hysteresis
$\mathbf{k_{STO}}$: Maximum Storage Rate [T^{-1}]
$\mathbf{k_{ads}}$: Maximum Adsorption Rate [T^{-1}]
$\mathbf{k_H}$: Maximum Specific Hydrolysis Rate [T^{-1}]
$\mathbf{K_S}$: Half Saturation Constant [$M.L^{-3}$]
$\mathbf{K_{S1}}$: Half Saturation Constant for Direct Growth [$M.L^{-3}$]
$\mathbf{K_{S2}}$: Half Saturation Constant for Growth on Stored Polymers [$M.L^{-3}$]
KST : Kinetic Selection Theory
$\mathbf{K_{STO}}$: Half Saturation Constant for Storage [M COD.$(M\ COD)^{-1}$]
$\mathbf{K_X}$: Half Saturation Constant for the Hydrolysis
MLSS : Mixed Liquor Suspended Solid [$M.L^{-3}$]
NADH : Nicotinamide Adenine Dinucleotide
NMR : Nuclear Magnetic Resonance
OLR : Organic Loading Rate
OUR : Oxygen Utilization Rate [$M.L^{-3}.T^{-1}$]
$\mathbf{OUR_{dec}}$: Oxygen Utilization Rate for Endogenous Decay [$M.L^{-3}.T^{-1}$]
$\mathbf{OUR_{gro}}$: Oxygen Utilization Rate for Growth [$M.L^{-3}.T^{-1}$]
$\mathbf{OUR_{respXsto}}$: Oxygen Utilization Rate for Respiration of X_{STO} [$M.L^{-3}.T^{-1}$]
$\mathbf{OUR_{sto}}$: Oxygen Utilization Rate for Storage [$M.L^{-3}.T^{-1}$]
PAO : Phosphorus Accumulating Organism
PHA : Polyhydroxyalkanoate
PHB : Poly-β-hydroxybutyrate
ppGpp : Guanosine Tetraphosphate
PSS : Protein Synthesis System

$q_{p(g)}$: Specific Storage Product Formation Rate[M.(M COD.T)$^{-1}$]
$-q_{p(g)}$: Specific Storage Product Consumption Rate [M.(M COD.T)$^{-1}$]
$-q_S$: Specific Substrate Removal Rate [M.(M COD.T)$^{-1}$]
RNA : Ribonucleic Acid
SBR : Sequencing Batch Reactor
S_I : Soluble Inert COD Concentration [M COD.L^{-3}]
S_O : Dissolved Oxygen Concentration [M O_2.L^{-3}]
SolS : Soluble Starch
SRT : Sludge Retention Time [T]
S_S : Readily Biodegradable COD Concentration [M COD.L^{-3}]
SVI : Sludge Volume Index
TCA : Tricarboxylic Acid Cycle
ThOD : Theoretical Oxygen Demand
VFA : Volatile Fatty Acid
VSS : Volatile Suspended Solid
WWTP : Wastewater Treatment Plant
X_{ads} : Adsorbed COD Concentration [M COD.L^{-3}]
X_H : Heterotrophic Biomass Concentration [M COD.L^{-3}]
X_I : Particulate Inert COD Concentration [M COD.L^{-3}]
X_P : Inert Microbial Product Concentration [M COD.L^{-3}]
X_S : Slowly Biodegradable COD Concentration [M COD.L^{-3}]
X_{STO} : Storage Polymer Concentration [M COD.L^{-3}]
X_{TS} : Total Suspended Solids Concentration [M COD.L^{-3}]
Y_H : Heterotrophic Growth Yield [M COD.(M COD)$^{-1}$]
Y_{H1} : Yield Coefficient for Direct Growth [M COD.(M COD)$^{-1}$]
Y_{H2} : Yield Coefficient for Growth on Stored Polymers [M COD.(M COD)$^{-1}$]
Y_{obs} : Heterotrophic Observed Yields [M COD.(M COD)$^{-1}$]
Y_{PX} : Yield of Biomass on PHB [M COD.(M COD)$^{-1}$]
Y_{SP} : Yield of PHB on acetate [M COD.(M COD)$^{-1}$]
Y_{STO} : Storage Yield [M COD.(M COD)$^{-1}$]
Y_{SX} : Yield of Biomass on Acetate [M COD.(M COD)$^{-1}$]
μ_{Hmax} : Maximum Spesific Growth Rate for Heterotrophs [T^{-1}]
μ_{H1} : Maximum Spesific Growth Rate for Direct Growth [T^{-1}]
μ_{H2} : Maximum Spesific Growth Rate for Secondary Growth [T^{-1}]
γ_K : Collinearity Index
$\tilde{\lambda}_K$: Minimum Eigenvalue of the Normalized Subset Matrix
δ_j^{mabs} : Sensitivity Measure
δ_j^{msqr} : Sensitivity Measure

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GROWTH AND STORAGE KINETICS OF ACTIVATED SLUDGE SYSTEMS OPERATED WITH VARIOUS CARBON SOURCES FOR DIFFERENT SLUDGE AGES

SUMMARY

Mechanisms of substrate utilization by mixed microbial cultures such as activated sludge are quite important because it affects the oxygen uptake rate under aerobic conditions and process performance, especially for nitrogen removal. Depending on operating conditions, substrate may be directly used for microbial growth as it is traditionally conceived, or stored as internal polymers. Experimental observations indicated that the conversion of external substrate to storage polymers becomes the primary process for the removal of the substrate in activated sludge systems under transient conditions sustaining sequential feast and famine conditions (van Loosdrecht et al., 1997). Intermittent substrate feeding, either as a pulse or in a relatively short period creates a feast period where available substrate in the reactor largely exceeds the metabolic requirements of the biomass. During this period, external substrate is converted into internal storage products. In the following famine period with no external substrate availability, stored polymers serve as substrate for microbial growth (van Loosdrecht et al., 1997; Majone et al., 1999). In other words, when the duration of excess external substrate availability is not enough for the adaptation of the physiological state to fast availability of substrate, synthesis rate of storage polymer is stimulated. On the contrary, when the excess external substrate is available for a long period, the specific growth rate of biomass will be increased and the synthesis rate of storage polymer will be decreased. Therefore, high storage capacity has been observed in pulse fed activated sludge systems. Dionisi et al. (2001) argued that the feast period should be no more than 25-30% of overall reaction period in order to have a strong storage response.

Recognition of storage as a means of substrate removal led the way to a number of questions requiring clarifications. (i) Conversion of substrate to storage polymers was also observed under continuous feeding: Majone et al. (1996) reported significant PHB storage in two parallel continuously mixed reactors (CSRTs) operated at a sludge age of 3 days, one with continuous and the other with intermittent feeding of acetate. Van Loosdrecht and Heijnen (2002) reported that a PHB pool was sustained in the biomass, although the amount of PHB generation was affected by the change in the feeding period. (ii) Direct growth on external substrate and storage could occur simultaneously in systems fed with a simple substrate such as acetate (Krishna and van Loosdrecht, 1999) or complex substrates such as starch and peptone (Karahan-Gül et al., 2003; Orhon et al., 2009; Katipoğlu et al., 2010). (iii) Different microbial groups could perform substrate storage: In fact, filamentous and floc forming bacteria sustained under different feeding regimes could both store PHB (Majone et al., 1996). The storage capability of the filamentous bacteria was later confirmed in a similar study of the same group Beccari et al. (1998). Similarly, comparable PHB generation rates were reported in bad settling and well settling

biomass sustained under continuous and pulse feeding conditions, respectively (Martins et al., 2003).

Furthermore, the type and extent of biomass response depend on its microbial composition and on the physiological state of the mixed culture, which are in turn defined by previous "history" of the biomass, i.e. the operating conditions (sludge age, etc.). Beun et al., (2000) suggested that the PHB synthesis process should be determined with respect to the operating conditions. Krishna and van Loosdrecht (1999) reported that the ratio of storage to the overall substrate removal was reduced when the sludge age of an SBR system was lowered from 9.5 days to 3.8 days. In fact, the growth response is a more general phenomenon; however, storage response is triggered as the alternative mechanism in the case of slow growth response with respect to the dynamic conditions (Daigger and Grady, 1982). For example, as the microbial growth rate is faster at the lower SRTs, the magnitude of the storage will be lower compared to the activated sludge acclimated to same conditions at the higher SRT (Çığgın et al., 2011). Therefore, substrate removal mechanisms of activated sludge systems require appropriate consideration of related operating conditions.

The substrate removal mechanisms also depend on the type of the substrate. Differently from the readily biodegradable substrate such as acetate and glucose used as model substrate in many studies (Beun et al., 2000; Dirks et al., 2001), the slowly biodegradable substrate needs to be hydrolyzed to simple soluble compounds before utilization by the microbial community. As the slowly biodegradable substrate represents the major fraction of the complex organics in domestic sewage and industrial wastewaters (Orhon and Ubay Cokgor, 1997), it is important to gain knowledge on the fate of the slowly biodegradable substrate for elucidating the substrate utilization processes in activated sludge systems. Starch has been selected as high molecular weight slowly biodegradable substrate in similar studies (Karahan et al., 2006) as it is stored as glycogen instead of the well tested PHB storage.

In this context, the objective of this study was to investigate the utilization mechanisms of acetate, starch and mixture of these carbon sources at two different sludge ages under different aerobic feeding conditions by activated sludge. As well as substrate removal mechanisms, differences in microbial composition were determined by means of Fluorescence in Situ Hybridization (FISH). This way, the study intended to cover all the major factors, i.e. feeding regime; sludge age and microbial composition on resulting mechanism of substrate utilization. The modelling studies carried out for the evaluation of the stoichiometry and kinetic of activated sludge systems acclimated under different operating conditions indicated the consumption of starch via different microbial processes depending on the sludge age, although feeding regime had an effect only on storage rate of microorganisms. The experimental results obtained with different carbon sources revealed the mechanistic and microbial differences between the storage of the PHB and glycogen in terms of the stoichiometry, kinetic and microbial composition of activated sludge systems acclimated under different operating conditions. The comparison of the substrate removal performances under mixed substrate with under single substrate environments, observed with ^{13}C Nuclear Magnetic Resonance (NMR) studies, showed the adverse effect of starch on the acetate removal mechanism.

DEĞİŞİK KARBON KAYNAKLARINDA ÇALIŞTIRILAN AKTİF ÇAMUR SİSTEMLERİNDE FARKLI ÇAMUR YAŞLARINDA ÇOĞALMA VE DEPOLAMA KINETİĞİ

ÖZET

Aktif çamur gibi karışık mikrobiyal kültürler tarafından gerçekleştirilen substrat tüketimine ait mekanizmalar, aerobik koşullardaki oksijen tüketim hızını ve proses performansını etkilemeleri nedeniyle, özellikle azot arıtımı için, oldukça önemlidir. İşletme koşullarına bağlı olarak, substrat, geleneksel olarak kabul edildiği gibi, doğrudan mikrobiyal çoğalmada kullanılabilir veya içsel polimer olarak depolanabilir. Deneysel gözlemler, aktif çamur sistemlerinin ardışık feast ve famine (bolluk ve açlık) koşullarının oluşmasına neden olan transient (geçiş) koşullara maruz kalması durumunda, harici substratın depolama polimerlerine dönüştürülmesinin, substrat arıtımı için birincil proses olduğunu göstermiştir (van Loosdrecht et al., 1997). Kesikli substrat beslemesi, ister ani şekilde veya nispeten kısa bir süre içinde olsun, reaktörde mevcut substratın, biyokütlenin metabolik ihtiyaçlarının çok üzerine çıktığı bir feast periyodunu oluşturmaktadır. Bu periyot süresince, harici substrat içsel depolama ürünlerine dönüştürülmektedir. Takip eden, harici substratın ortamda mevcut olmadığı, famine periyotunda ise, depolanan polimerler mikrobiyal çoğalma için substrat olarak hizmet etmektedir (van Loosdrecht et al., 1997; Majone et al., 1999). Diğer bir deyişle, fazla harici substratın mevcut bulunduğu süre, fizyolojik durumun substratın hızla mevcut oluşuna adaptasyonu için yeterli olmadığı zaman, depolama polimeri sentez hızı artmaktadır. Tam tersine, fazla harici substrat uzun bir süre mevcut olduğunda, biyokütlenin spesifik çoğalma hızı artacak ve depolama polimerinin sentez hızı azalacaktır. Bu nedenle, ani beslenen aktif çamur sistemlerinde yüksek depolama kapasitesi gözlemlenmiştir. Dionisi ve diğ. (2001) baskın bir depolama yanıtı alabilmek için, feast periyodunun, tüm reaksiyon periyodunun %25-30'undan fazla olmaması gerektiğini öne sürmüştür.

Depolamanın substrat arıtımı için bir yöntem olarak tanınması, açıklık getirilmesi gereken birkaç soruya yol açmıştır. (i) Substratın depolama polimerlerine dönüşmesi sürekli besleme altında da gözlenmiştir: Majone ve diğ. (1996), biri sürekli ve diğeri kesikli şekilde asetatla beslenmek üzere, çamur yaşı 3 günde çalıştırılan iki paralel tam karışımlı reaktörde önemli PHB (poli hidroksi bütirat) depolaması raporlamıştır. Van Loosdrecht ve Heijnen (2002), PHB üretiminin, besleme periyodundaki değişimden etkilenmesine rağmen, biyokütle içinde bir PHB havuzunun sürdürüldüğünü raporlamıştır. (ii) Asetat gibi basit bir substratla (Krishna and van Loosdrecht, 1999) veya nişasta ve pepton gibi karmaşık substratlarla (Karahan-Gül ve diğ., 2003; Orhon ve diğ., 2009; Katipoğlu et al., 2010) beslenen sistemlerde harici substrat üzerinde doğrudan çoğalma ve depolama bir arada gerçekleşebilir. (iii) Farklı mikrobiyal gruplar substrat depolamasını gerçekleştirebilir: Aslında, farklı besleme rejimleri altında tutulan filamentli ve flok yapan bakterilerin her ikisi de PHB depolayabilir (Majone et al., 1996). Filamentli bakterilerin depolama kabiliyeti

daha sonra aynı grubun, Beccari et al. (1998), benzer bir çalışmasında teyit edilmiştir. Benzer şekilde, sırası ile sürekli ve ani besleme koşullarında tabi tutulan kötü çökelen ve iyi çökelen biyokütlelerde benzer PHB üretim hızları rapor edilmiştir (Martins ve diğ. 2003).

Buna ek olarak, biyokütle yanıtının tipi ve boyutu, karşılıklı olarak biyokütlenin önceki "geçmişi", yani işletme koşulları (çamur yaşı, vb.), tarafından belirlenen, mikrobiyal kompozisyonuna ve karışık kültürün fizyolojik durumuna bağlıdır. Beun ve diğ., (2000) PHB sentezleme prosesinin işletme koşullarına bağlı olarak belirlenmesini önermektedir. Krishna ve van Loosdrecht (1999), bir ardışık kesikli reaktör sisteminin çamur yaşı 9.5 günden 3.8 güne indirildiğinde depolamanın tüm substrat arıtımına oranının düştüğünü raporlamıştır. Aslında, çoğalma yanıtı daha genel bir hadisedir, bununla birlikte, depolama yanıtı dinamik koşullara bağlı oluşan yavaş çoğalma yanıtı durumunda alternatif mekanizma olarak tetiklenmektedir (Daigger ve Grady, 1982). Örneğin, düşük çamur yaşlarında mikrobiyal çoğalma hızı daha hızlı olduğundan, depolamanın büyüklüğü, aynı koşullara aklime edilmiş daha yüksek çamur yaşındaki aktif çamura kıyasla daha düşük olacaktır (Çığgın ve diğ., 2011). Bu nedenle, aktif çamur sistemlerinin substrat arıtımı mekanizmaları ilgili işletme koşullarının uygun şekilde dikkate alınmasını gerektirmektedir.

Substrat arıtım mekanizmaları substratın türüne de bağlıdır. Birçok çalışmada (Beun ve diğ., 2000; Dirks ve diğ., 2001) model substrate olarak kullanılan asetat ve glikoz gibi biyolojik olarak kolay ayrışan substrattan farklı olarak, biyolojik olarak yavaş ayrışan substrat mikrobiyal topluluk tarafından tüketilmeden önce basit çözünmüş bileşiklere hidrolize olması gerekmektedir. Biyolojik olarak yavaş ayrışan substrat, evsel atıksu ve endüstriyel atıksudaki karmaşık organiklerin başlıca fraksiyonunu temsil ettiğinden (Orhon ve Ubay Cokgor, 1997), aktif çamur sistemlerinde substrat tüketimini açıklayabilmek için, biyolojik olarak yavaş ayrışan substratın nihayeti üzerinde bilgi edinmek önemlidir. Nişasta, detaylı bir şekilde araştırılmış PHB yerine glikojen olarak depolanması nedeniyle, yüksek moleküler ağırlıkta biyolojik olarak ayrışabilen substrat olarak benzer çalışmalarda seçilmiştir (Karahan ve diğ., 2006).

Bu bağlamda, bu çalışmanın amacı, asetat, nişasta ve bu karbon kaynaklarının karışımının, iki farklı çamur yaşında, değişik aerobik besleme koşullarında aktif çamur tarafından tüketim mekanizmalarının araştırılmasıdır. Substrat arıtım mekanizmalarının yanı sıra, mikrobiyal kompozisyondaki değişiklikler Flüoresanlı yerinde hibritleşme (FISH) tekniği ile belirlenmiştir. Bu şekilde, bu çalışma substrat tüketim mekanizması üzerinde etkili tüm başlıca faktörleri yani besleme rejimi, çamur yaşı ve mikrobiyal kompozisyon, kapsamayı planlamıştır. Değişik işletme koşullarında aklime edilen aktif çamur sistemlerinin stokiyometrisi ve kinetiğinin değerlendirilmesi için yürütülen modelleme çalışmaları, besleme rejiminin sadece mikroorganizmaların depolama hızına etki etmesine rağmen, çamur yaşına bağlı olarak nişastanın değişik mikrobiyal proseslerle tüketildiğini göstermiştir. Değişik karbon kaynaklarından elde edilen deneysel sonuçlar, farklı işletme koşullarına aklime edilmiş aktif çamur sistemlerinin stokiyometri, kinetik ve mikrobiyal kompozisyonları bakımından, PHB ve glikojen depolaması arasındaki mekanistik ve mikrobiyal farklılıkları açığa çıkarmıştır. Karışık substrat ortamında ve tekil substrat ortamında, yapılan ^{13}C Nükleer Manyetik Rezonans (NMR) çalışmaları ile gözlenen substrat arıtım performanslarının kıyaslanması, nişastanın asetat arıtım mekanizması üzerindeki karşıt etkisini göstermiştir.

1. INTRODUCTION

1.1 Purpose of the Thesis

In biotechnological applications, accurate determination of each kinetic parameter is of great importance for appropriate reactor design and process control. The success in the determination of the kinetic and stoichiometric parameters may suffer from many factors reflecting alteration of microbiological properties of the microbial culture. These factors are namely (1) the culture history (2) feeding type/pattern of substrates and (3) failure to recognize other subsidiary microbial processes such as storage, overflow mechanism etc., taking place simultaneously together with growth under different environmental conditions.

A numerous studies have been performed for understanding the mechanistic behaviour of the microorganisms in activated sludge systems. The growth response is a more general phenomenon; however, storage response is triggered as an alternative mechanism in the case of slow growth response with respect to transient conditions. Due to time or space dependent changes in wastewater treatment plants (WWTPs), bacteria frequently encounter with transient conditions. The experiments conducted in activated sludge systems, operated under dynamic conditions, indicate that storage of internal polymers is usually the main mechanism for the removal of readily biodegradable carbon sources.

After storage phenomena was recognized as one of the main process for carbon removal in activated sludge systems, Activated Sludge Model No. 3 (ASM3) (Gujer et al., 2000) was introduced for determination of the kinetic and stoichiometric coefficients in storage process. According to ASM3, a new phenomena namely storage has been discussed. In ASM3, the removal of external substrate was explained with storage: all readily biodegradable substrate is first being stored inside the cells and then storage products are used for growth without addition of external substrate under both aerobic and anoxic conditions.

There were several approaches for the modelling of storage. In literature, metabolic modelling approach became important, when dynamic conditions were taken into account. The studies, which used metabolic modelling approach, have clarified storage different than ASM3. Although carbon source was assumed to be used for storage in ASM3, the metabolic modelling studies have proven simultaneous presences of the direct growth and storage mechanisms.

The presence and relative distributions of storage polymers in substrate removal relies on the internal metabolism mechanisms of carbon source. Glycogen is mostly formed when the primary substrate is glucose or a compound that can be converted into pyruvate with an increase in degrading power; for example, different carbohydrates, glycerol, or proteins. On the other hand, Polyhydroxyalkanoate (PHA) is directly formed from the central metabolite acetyl-Coenzyme A (Acetyl-CoA) (Doi, 1990), which is produced in the degradation of volatile fatty acid (VFA)s. PHA is considered as the most important storage polymer, because VFAs are the major soluble substrate constituents in wastewaters. In extensive studies it has been proven that if the system is fed with only acetate, the formed PHA is Poly-β-hydroxybutyrate (PHB).

The substrate removal mechanisms also depend on the type of the substrate. Differently from the readily biodegradable substrate, the slowly biodegradable substrate needs to be hydrolyzed extracellularly to soluble substrate before consumption for growth by bacteria. As the slowly biodegradable substrate represents the major fraction of the complex organics in domestic sewage and industrial wastewaters, it is important to gain knowledge on the fate of the slowly biodegradable substrate as well as the readily biodegradable one for elucidating the microbial processes which take place in activated sludge systems. Within this framework, the goal of this study is to observe and elaborate the kinetic and stoichiometric coefficients in activated sludge systems operated under different operating conditions with different carbon sources.

For this purpose, in first part of the thesis, the substrate removal mechanism was evaluated for readily biodegradable substrate in terms of feeding pattern, sludge retention time, organic loading rate and inoculum. The studies performed under different sludge retention times and different feeding patterns have also been carried out for slowly biodegradable substrate. The carbon sources stored as different storage

polymers were selected for comparative evaluation of the storage mechanism. The microbial composition of systems operated under different conditions were determined and compared.

After general knowledge on the carbon removal processes under different operating conditions had been gained, experimental results obtained from activated sludge samples acclimated to different carbon sources were modelled in the second part of thesis.

As a final part of the thesis, the activated sludge systems were acclimated to the mixed carbon source for evaluation of the interaction between the removal processes of different carbon sources. For this purpose, the interaction in the substrate removal mechanisms was investigated with comparison of the substrate removal performances, microbial characterization and storage polymer formation. Finally, metabolic pathways were evaluated in systems operated either with single carbon source or with multiple carbon sources.

1.2 Scope and Outline of Thesis

The scope of this thesis was to gain a better understanding on how much transient condition can simultaneously affect both the mechanism of substrate removal (i.e. the relative role of storage and growth) and the microbial composition of activated sludge. Hence, the effect of different feeding patterns was investigated by running two SBRs in parallel, with pulse vs. continuous feeding, respectively. While carbon source was given during 1 minute in pulse feeding, continuous feeding was applied to activated sludge with feeding the same amount of carbon source during the reaction period.

Moreover, these two parallel SBRs were operated at two sludge retention times (SRTs) and two organic loading rates (OLRs) started up with fresh inoculum, in order to understand whether the effect of feeding pattern is more or less important compared to SRT and OLR. Molecular in situ detection methods (FISH) and microscopic staining procedures (Nile blue) were used to investigate the microbial composition, in combination with quantitative determination of rate and yield of storage phenomena under all investigated conditions.

In particular, the experimental activity was designed to understand whether the increase of storage under dynamic conditions is either induced by changes of microbial composition or by physiological adaptation of the given microbial composition to dynamic conditions. Moreover, the latter mechanism was further investigated to understand how much the physiological adaptation is a short-term effect or if it requires longer acclimation to dynamic feeding. Therefore, the effect of a shift from continuous to dynamic conditions was investigated by two subsequent runs in a sequencing batch reactor (SBR) under aerobic conditions. Moreover, single disturbance of usual feeding conditions was imposed to each acclimated biomass to understand short-term effects and distinguish them from long-term effects after long acclimation.

The studies were carried out with carbon sources which have a different nature. After the extensive studies were completed with readily biodegradable substrate, namely acetate, starch was selected as a high molecular weight and slowly biodegradable substrate in many studies, because it is stored as glycogen instead of the well recognized PHB.

The aim of performing studies with starch was to explore the fate of the slowly biodegradable substrate at different sludge retention times and feeding patterns in activated sludge system under aerobic conditions. For this purpose, the activated sludge was acclimated to soluble starch (SolS) in parallel SBRs. The first purpose was to investigate the removal mechanism of starch and related microbial composition at two different SRTs as 8 and 2 days under aerobic conditions. The experiments were carried out in parallel SBRs operated under two different feeding patterns in order to promote the storage and direct growth mechanisms at pulse and continuously fed SBRs, respectively. Both the substrate removal mechanisms and the differences in microbial composition related to the SRT and feeding pattern were detected with Fluorescence in Situ Hybridization (FISH). Secondly, the results were compared with the previous study carried out with acetate under the same operating conditions for observation of kinetic and microbial differences between the storage of the PHB and glycogen.

Finally, aerobic metabolism of mixed carbon sources in an activated sludge process were evaluated as a function of the sludge retention time and feeding pattern, referring primarily to the role of storage compounds under dynamic conditions. Since

the different storage polymers were originated from different metabolites, this study was carried out with the mixture of two different carbon sources: acetate which is one of the important substrates in domestic wastewater and which is usually stored as PHB and starch which requires hydrolysis by extracellular enzymes prior to its consumption by biomass and is stored as glycogen. While the relative contribution of the direct growth and storage process to substrate removal were investigated by traditional chemical analyses, the metabolic pathways involved in the formation of storage polymers from these substrates were investigated by means of Carbon Nuclear Magnetic Resonance (^{13}C-NMR) analysis.

2. DYNAMICS OF ACTIVATED SLUDGE PROCESSES

2.1 Activated Sludge Model No. 1 (ASM1)

The International Association on Water Quality (IAWQ, formerly IAWPRC) was established in 1983 for the development and application of practical models in activated sludge systems. After extensive studies, Activated Sludge Model No.1 (ASM1) was developed by IAWQ Task Group with mathematical modelling for design and operation of biological wastewater treatment systems (Henze et al., 1987). Although this model has been extended considering some important processes such as biological phosphorus removal (ASM2, Henze et al,. 1994) and storage phenomena (ASM3, Gujer et al., 2000), ASM1 have still provided basis for the design and modelling of activated sludge systems. In other words, it is essential to have comprehensive knowledge on this first conceptual model ASM1, to understand all basic processes of activated sludge. ASM1 generally takes into consideration these three components: biomass, substrate and oxygen. The basic components and process of ASM1 for carbon oxidation is shown in Table 2.1.

Table 2.1: Processes for carbon oxidation described in ASM1.

Component / Process	S_O	S_S	X_S	X_P	X_H	Process rate
Growth	$-\frac{1-Y_H}{Y_H}$	$-\frac{1}{Y_H}$			1	$\hat{\mu}_H.\left(\frac{S_S}{K_S+S_S}\right)\left(\frac{S_O}{K_O+S_O}\right)X_H$
Hydrolysis		1	-1			$k_h\left(\frac{X_S/X_H}{K_X+X_S/X_H}\right)\left(\frac{S_O}{K_O+S_O}\right)X_H$
Decay			$1-f_P$	f_P	-1	$b_H.X_H$

As seen from the simplified representation of ASM1, this model integrates two stoichiometric and six kinetic parameters to describe the utilization of wastewater by heterotrophic microorganisms. The biodegradable substrate in influent wastewater consists of two fractions: readily and slowly biodegradable substrate. Although, existing readily biodegradable substrate (S_S) is assumed to consist of simple

molecules which are able to pass through the cell wall and immediately be used for microbial growth, the slowly biodegradable substrate (X_S), which consists of larger complex molecules, is adsorbed by activated sludge, and then broken down by extracellular enzymes (hydrolysis) before being transferred through the cell wall and used for metabolism. The processes of simplified ASM1 can be described as:

- *Growth*: A fraction of the readily biodegradable substrate is used for growth of heterotrophic biomass and oxidized for energy requirement of the cell. The growth is modelled by using Monod kinetics. Both the concentration of S_S and dissolved oxygen (S_O) may be rate limiting for growth process. This process is generally the main contributor to the production of new biomass and removal of substrate.

- *Hydrolysis*: Slowly biodegradable substrate (X_S) is broken down to readily biodegradable substrate by extracellular enzymes, and then the latter one is available to the organisms for growth. This process is modelled on the basis of surface reaction kinetics. Therefore, the rate is expressed by first-order kinetic with respect to the present heterotrophic biomass.

- *Decay*: This process is modelled according to the death-regeneration hypothesis. Inert microbial product (X_P) is generated during the lysis of heterotrophic microorganism (X_H) with fraction f_P. The remaining fraction of ($1-f_P$) is transformed into X_S. It is assumed that carbon is not removed during this transformation and as a consequence no electron acceptor is utilized.

The main stoichiometric parameters represented in simplified ASM1 are the heterotrophic yield coefficient (Y_H) and fraction of biomass, leading to particulate product (f_P). The coefficient f_P, which is consumed as inert particulate product after decay process, is affected by the death-regeneration concept. If the decay process is modelled as endogenous decay, this value is usually assumed to be approximately 0.2. However, the recycling of biomass by death-regeneration, results in significantly lower f_P value in order to reach the same amount of particulate inert mass. Y_H is a stoichiometric constant known as observed growth yield. It is simply defined as weight of new biomass produced from the growth-limiting substrate. The yield term indicates the amount of substrate, which is used both for new biomass production and also for energy generation to carry out biosynthetic reactions. Thus, both

theoretical and experimental data show that Y_H is not a biological constant and depends on the carbonaceous substrate, the efficiency of oxidative phosphorylation, the anabolic and catabolic pathways utilized, the presence of preformed monomers, and the nitrogen source (Stouthamer, 1979; Stouthamer and Van Verseveld, 1985). The Y_H is an important parameter, as it effects estimation of sludge production and oxygen demand; it also has an impact on kinetic parameters. As the Y_H highly depends on the biomass, it is assumed to be rather constant in different municipal Wastewater Treatment Plants (WWTPs). The typical value of Y_H is given as 0.67 for municipal wastewaters in ASM1.

Kinetic parameters are highly dependent on the nature of the substrate. Therefore, it is necessary to determine these parameters by case specific analyses. The interaction of different processes makes the determination of decay coefficient (b_H) difficult related to the death regeneration concept. The maximum specific growth rate (μ_{Hmax}) depends on the sludge age and therefore depends on both characteristics of wastewater and operation of the WWTPs. The half saturation coefficient for heterotrophic biomass (K_S) also depends on the operation of the WWTPs, due to the dependency of the floc size and structure on diffusion of substrate to the cell and thereby on the apparent value of saturation coefficients (Jeppsson, 1996). The hydrolysis process in ASM1 is used to describe the degradation of slowly biodegradable organic substance originating from the influent carbon source and from internal turnover of substrate in the death regeneration cycle. In ASM1, the kinetic parameters of hydrolysis process, which are the maximum specific hydrolysis rate (k_h) and half saturation coefficient for hydrolysis (K_X) were suggested as model parameters that have to be adjusted during model calibration procedure.

Structured and unstructured models were introduced by several researchers for the processes taken place in WWTPs. In unstructured models, the activity of the biomass is represented only by biomass concentration without considering the activity of biomass. These models might provide realistic descriptions, if there are no changes in environmental conditions or if only carbon removal is taken into consideration (Roels, 1983). In these models, the biomass is assumed at pseudo steady state like in ASM1, in which the endogenous decay metabolism is assumed as constant-regarding death-regeneration approach (Majone et al., 1999).

However, these approaches do not reflect the realistic environmental conditions. Therefore, the structured models regarding the dynamic response of the microorganisms as well as their concentration have to be taken into account due to the physical adaptation and population dynamics affected by the environmental changes (Roels, 1983). For example, the change in operating conditions of microbial culture leads to fundamental changes in metabolic pathways. Hence, the adaptation of biomass to changing environmental conditions determines its dynamic response. A thorough understanding of microbial adaptation to environment provides the basis of understanding dynamic response. Daigger and Grady (1982) were the first authors who used structured approach to model the metabolic status of biomass in activated sludge processes. They noted that the biomass usually grows under transient (unsteady) conditions in activated sludge processes due to unavoidable fluctuations observed in feed flow rate, feed composition and temperature. Therefore, dynamic response of the microbial culture under transient conditions should be considered for realistic prediction of the performance of biochemical unit processes.

2.2 Dynamic Response of Activated Sludge

Physiological adaptation of the biomass is a well known behaviour of activated sludge which is resulted from the changes in biochemical pathways of microorganisms. The physiological state of the microbial cultures, which is a function of culture history, is adjusted by a number of biochemical components (RNA, DNA, protein, carbohydrate, and lipid), specific enzymes, and metabolic intermediates (Daigger and Grady, 1982). Dynamic response of a culture regarding physiological adaptation is evaluated in two general classes: growth responses and storage responses. The occurrence of these two processes is mainly explained by specific growth rate of microorganisms (Daigger and Grady, 1982).

2.2.1 Growth response

The growth response observed under transient conditions is explained with two theories. The theory of Growth Rate Hysteresis (GRH) is explained with the tendency of biomass to growth under a lower rate than the maximum rate, when the substrate concentration fluctuates. In other words, during the increasing substrate concentration, the specific growth rate will be less than predicted, however, during

the decreasing substrate concentration the specific growth rate will be greater (Perret, 1960). The second type of growth response, called Available Reaction Potential (ARP) is explained with the growth increasing ability of microbial culture and substrate removal rates rapidly during a transient response (McLellan and Busch, 1969).

Microbial cultures are capable of having both GRH and ARP under dynamic conditions (Daigger and Grady, 1982). In case of unexpected increase in substrate concentration, ribonucleic acid (RNA) content, enzyme levels, and concentration of intracellular metabolites decrease and culture is not able to grow at its maximum rate. Consequently, the culture has a limited ability to increase its specific growth rate immediately (ARP). Furthermore, because of the gradually increase in the levels of RNA, enzymes, and intracellular metabolites with time, the specific growth rate of the culture will increase and eventually reach the maximum value under particular conditions (GRH). RNA plays a particularly important role during a transient response due to protein synthesis. Decreases in the RNA content will limit the ability of protein synthesis, thereby leading to GRH response. Moreover, if RNA content does not decrease to the same extent as the specific growth rate, cultures grown at low specific growth rates might contain extra RNA which could be used immediately during ARP response. The "'constant efficiency hypothesis"' theory proposed by Maaloe and Kjeldgaard (1966) stated that the RNA was always synthesizing protein at its maximum rate and the rate of protein synthesis was regulated by controlling the amount of RNA. Due to decrease in RNA level concomitantly with specific growth rate, “constant efficiency hypothesis” demonstrates the existence of GRH response. The behaviour of microorganisms explained with these hypotheses depends on the changes in the RNA level of the culture which results from physiological adaptations of culture to different environmental conditions.

2.2.2 Storage response

The occurrence of storage during transient conditions is consistent with the principles of physiological adaptation. Storage has been explained as the survival mechanism when microorganism is prevented from growing at its maximum rate (Van Looshdrect et al., 1997). Although the substrate is transported into the cell, the biosynthetic growth limitations may prevent the consumption of all energy. This leads to an increase in energy charge which, in turn, leads to storage of the substrate.

In this way, favourable growth conditions are established to face against this highly dynamic environment in activated sludge processes.

As a result of highly dynamic feeding conditions in activated sludge systems, microorganisms are subjected to feast-famine regime (Van Loosdrecht et al., 1997). Feast regime is defined as the period in which microorganisms have external substrate. On the contrary, the famine regime is defined as the period in which the external carbon source is unavailable for growth. During this famine period, intracellular storage polymers are used as an internal carbon source (Van Loosdrecht et al., 1997).

In metabolic concept, the observation of the storage response under transient conditions was explained with two theories which are RNA limiting theory and metabolic flux (Grady et al., 1996). Microorganisms have an ability to optimize the growth process through the adaptation of macromolecular composition to imposed environmental conditions (Herbert, 1961). The changes in macromolecular composition within the cell are recognized as physiological adaptation (Daigger and Grady, 1982). Therefore, physiological adaptation has been studied as a key factor for understanding and prediction of transient response of microbial cultures. The physiological adaptation was explained with the RNA limiting theory (Grady et al., 1996). According to this theory, cellular macromolecules play an important role in the overall response of a microbial culture to transient conditions. To explain the RNA limiting theory, Grady et al. (1996) introduced the protein synthesis system (PSS), which is the largest single component of the biomass.

Biomass adapts PSS to new environmental conditions so that the cell maximizes the use of the monomers and energy for synthesis of new cell. Physiological state of cell impacts the substrate removal rate, because PSS determines the synthesis rate of catabolic enzymes. Therefore, there is a direct relationship between the size and rate of PSS and specific growth rate of culture (Bremer and Dennis, 1987).

Proteins are synthesized in two steps. During the first step, transcription, the RNA polymerase transcribes the DNA code to produce mRNA. In the second step translation, ribosomes translate the RNA to produce proteins. Therefore, PSS is directly related to RNA levels in the cell, which causes limitation of microbial growth rate (Herbert 1958; Koch 1970). In other words, a decrease in RNA content

indicates a reduction in protein synthesis and maximum growth rate (Daigger and Grady, 1982).

When the substrate is present, most of the RNA and enzymes are induced and this induction may cause to an imbalance between substrate uptake and consumption by the cells. Cell compensates this imbalance by storing the excess substrate as a storage polymer (Van Loosdrecht et al., 1997). Although, the higher amount of RNA is needed for growth at maximum rate, the storage of substrate as an internal storage product requires a relatively lower amount of enzymes. In general, the PSS, RNA level and growth rate of cells are highly unpredictable for bacteria cultivated under different growth conditions, because of the different physiological states of the cells. For example, the microbial systems adapted to faster growth conditions (lower sludge retention time) adjust their PSS to accommodate higher levels of external substrate for growth. On the contrary, biomass adjusted to lower growth (higher sludge retention time) conditions leads to storage of external substrate (Daigger and Grady, 1982).

Metabolic flux is based on mass balance and stoichiometry of metabolic pathways (Cortassa et al., 1995). The overall metabolic processes are divided into anabolic and catabolic pathways. In metabolism, central catabolic pathways (glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, oxidative phosphorylation) provide the key intermediary precursors of monomer synthesis (amino acids, lipids, sugars, nucleotides) for the main macromolecular components (proteins, lipids, polysaccharides, nucleic acids). The production rate and yield of a metabolite are ultimately limited by the ability of cells to channel the carbon flux from central catabolic pathways to main anabolic routes which is leading to biomass synthesis (Cortassa et al., 1995; Liao et al., 1996). Growth occurs as a result of the dynamic balance between synthesis and degradation of macromolecular components. In case of an imbalance between catabolic and anabolic fluxes, the main anabolic routes are interrupted and this leads to redirection of the metabolic fluxes toward the production of storage polymers. Thus, metabolic flux coordination is an expression of the dynamic response of the cell.

The microorganism’s ability to channel carbon flux from central catabolic pathways to main anabolic routes can be interrupted thermodynamically or kinetically. The growth limitation factor can disturb the flux through main anabolic routes, because of

limitation in the chemical potential of the growth reaction thermodynamically. Kinetic obstruction of the carbon flux is caused by the excess catabolic enzymes. In this case the energy might be released faster than its consumption, which is leading to storage similarly observed in the RNA limiting theory. Consequently, the thermodynamic or kinetic interruption of the carbon flux through the main anabolic routes results in the observation of secondary metabolic route such as storage.

2.3 Modelling of Activated Sludge Systems under Dynamic Conditions

As a result of any changes in operating conditions, the level of intracellular components as well as the rates and the metabolic status of bacterial cells change. Therefore, it is necessary to determine the kinetic parameters for accurate description of the process exposed to new operational conditions or for accurate description of the new process configuration (Van Loosdrecht and Heijnen, 2002). The identification and interpretation of major biochemical reactions occurring in the specific conditions can be achieved with a modelling approach.

Models are essential to provide estimations of important but otherwise inaccessible process/physiological parameters, or to support the design of control strategies. Metabolic models, which incorporate a sequence of biochemical processes involving different model components, are based on the internal reactions that are combined with reaction stoichiometry and degree of reduction balances to derive the linear equation for description of involved metabolism (Roels, 1983).

2.3.1 Activated Sludge No.3 (ASM3)

In the last decade it has been recognized that bacterial populations adopt a survival strategy when exposed to feast and famine conditions, because of transient conditions. The production of reduced storage polymers is thought to serve as a nicotinamide adenine dinucleotide (NADH) overflow mechanism to control the redox state of heterotrophic cells during unbalanced growth conditions (Senior and Dawes, 1973; Van Niel et al., 1995). Effectively, the polymer acts as a buffer for the substrate that is taken up but not directly used for growth. In this way, growth of biomass can continue at a similar or slightly decreased rate in periods without external substrate supply. The whole mechanism enables the bacteria to maintain its

growth at a more or less constant or balanced rate and efficiently compete for substrate under dynamic substrate supply.

To take the endogenous decay phenomena into account, the Activated Sludge Model No.3 (ASM3) model was built on the basis of storage concept of readily biodegradable substrate by IWA Task Group (Gujer et al., 2000). In ASM3, it is hypothesized that all readily biodegradable substrate (S_S) is first stored as an internal storage product (X_{STO}), either directly or through preliminary hydrolysis, and then growth occurs only on internally storage polymers during the famine phase. ASM3 is one of the most referenced models for the quantitative analysis of activated sludge processes. The reaction kinetics and stoichiometry of ASM3 simplified for organic carbon removal is given in Table 2.2. The mechanisms participating in oxygen utilization are storage, growth, endogenous decay and respiration of storage products.

Table 2.2: The simplified ASM3 for carbon removal under aerobic conditions.

a) Components of Model

Component Process	S_O	S_S	$X_{STO,PHA}$	X_S	X_H
Storage of S_S	$-(1-Y_{STO})$	-1	Y_{STO}		
Growth on X_{STO}	$-\frac{1-Y_H}{Y_H}$		$-\frac{1}{Y_H}$		1
Endogenous respiration	$-(1-f_{ES}-f_{EX})$				-1
Respiration of X_{STO}	-1		-1		

b) Process rates of Model

Process	Process rate
Storage of S_S	$k_{STO}.\frac{S_S}{K_S+S_S}.X_H$
Growth on X_{STO}	$\mu_{H,PHA}.\frac{K_{S,GRO}}{K_{S,GRO}+S_S}.\frac{X_{STO}/X_H}{K_{STO}+X_{STO}/X_H}X_H$
Endogenous respiration	$b_H.X_H$
Respiration of X_{STO}	$b_{STO}.X_{STO}$

The full ASM3 model accounts for the existence of two groups of organisms (heterotrophic and autotrophic organisms) and attempts to simultaneously describe

the sludge production, nitrification and denitrification processes, as well as the storage of organic substrates, either through aerobic or anoxic storage of COD.

The main difference between ASM1 and ASM3 is the recognition of the importance of storage polymers in activated sludge processes. At the same time the decay process from ASM1 is replaced by an endogenous respiration process. The endogenous respiration concept originated from the observation that internal storage materials are used for maintenance purposes when the external substrate is depleted (Van Loosdrecht and Henze, 1999). A major difference for the wastewater characterization between ASM1 and ASM3 is that soluble (S_S) and particulate (X_S) biodegradable components in ASM3 are supposed to be differentiated with filtration over 0.45 μm membrane filters, whereas a significant fraction of the slowly biodegradable organic substrates (X_S) in ASM1 would be contained in the filtrate of the influent wastewater (Gujer et al., 2000). In addition, ASM3 contains nitrogen and alkalinity limitation for growth of microorganisms with description of ammonification kinetics by assuming constant N and COD ratio. The hydrolysis process has been very important in predicting the electron acceptor under aerobic and anoxic conditions.

Whereas the quantification of the kinetic parameters for hydrolysis process was difficult, ASM3 simplified hydrolysis process with only one process independently from electron acceptor. The model has also included the differences in the decay rates of nitrifiers under aerobic and anoxic conditions.

2.3.1.1 Components and processes of ASM3

ASM3 integrated seven soluble and six particulate components to characterize the wastewater and activated sludge. The first component is dissolved oxygen (So), which can directly be measured and it is subject to gas exchange. Inert soluble organic material (S_I) is a part of influent and may be produced by the hydrolysis of X_S. According to ASM3, readily biodegradable organic substrate (S_S) is first taken up by heterotrophic organisms and stored as X_{STO}, and then used for growth. S_S and S_I are approximately equal to the total soluble COD determined by filtration from 0.45 μm membrane filters.

Inert particulate organic matter (X_I) is a part of influent and may be produced as a result of biomass decay. Bacteria cannot directly use the colloidal and particulate

organic substrates, because they have high molecular weights. Therefore, slowly biodegradable substrates (X_S) must undergo hydrolysis before they are used by the biomass. The products of the hydrolysis are assumed to be readily biodegradable or soluble inert substrate. Heterotrophic organism (X_H) may grow both aerobically and anoxically. Hydrolysis does not consume any electron acceptor in ASM3. Active biomass is responsible for the hydrolysis of X_S and storage process. Internal storage products (X_{STO}) consist of PHA, glycogen etc., which is only defined as a functional component required for modelling, but it is not directly chemically identifiable. X_A represents autotrophic organisms responsible for nitrification. They oxidize the ammonium directly to nitrate; while nitrite is not considered in ASM3. Last component considered in model is the total suspended solids, X_{TS} that may be used for the modelling of volatile suspended solids by some special ratios. Twelve processes taken into account in ASM3 as summarized below:

- *Hydrolysis*: This process converts all of the slowly biodegradable substrates in the influent to available soluble substrate. This process is independent from electron acceptor and therefore different when compared to ASM1.
- *Aerobic storage of readily biodegradable substrate*: it is assumed that all readily biodegradable substrate is stored as storage products (X_{STO}), and then used for the growth of biomass. This conversion required energy which is produced by aerobic respiration in the form of ATP.
- *Anoxic storage of readily biodegradable substrate*: This process is similar to aerobic storage process, but energy requirement of this process is supplied by anoxic respiration. Due to lower amount of denitrifiers, the process rate is naturally smaller than aerobic storage rate.
- *Aerobic growth of heterotrophic organisms*: It is assumed that the stored products are used as substrate for the growth of heterotrophic microorganisms.
- *Anoxic growth of heterotrophic organisms*: This process is identical with aerobic growth, but it is based on anoxic respiration.
- *Aerobic endogenous respiration*: This process was explained as all forms of biomass loss and energy requirements which are not related to growth. The

process consists of decay, maintenance, endogenous respiration, lysis, predation, motility and death.

- *Anoxic endogenous respiration*: This process is similar to aerobic endogenous respiration, but occurs at a slower rate. Especially protozoa predation is considerably less active under anoxic conditions than under aerobic conditions.
- *Aerobic respiration of storage products*: X_{STO} decays together with biomass like endogenous respiration.
- *Anoxic respiration of storage products*: Denitrifying conditions are applied for the similar process of aerobic respiration of storage products.
- *Aerobic growth of autotrophic organisms*: Nitrifiers oxidize ammonium directly to nitrate as an intermediate component.
- *Aerobic endogenous respiration for autotrophic organisms*: Apart from description for nitrifiers, it takes place similar to aerobic endogenous respiration.
- *Anoxic endogenous respiration for autotrophic organisms*: It is similar to aerobic endogenous respiration for autotrophic organisms, but it occurs at a slower rate.

2.3.1.2 Process stoichiometry

The net (true) yields of heterotrophic biomass (X_H) produced per unit of readily biodegradable substrate (S_S) removed in ASM3 are found from following equations.

$$Y_{net,O2} = Y_{STO,O2}.Y_{H,O2} \quad \textbf{(2.1)}$$

$$Y_{net,NO} = Y_{STO,NO}.Y_{H,NO} \quad \textbf{(2.2)}$$

All stoichiometric parameters of ASM3 are identified together with their units and a typical value in Table 2.3. The composition of all organic fractions relative to Theoretical Oxygen Demand (ThOD) is assumed be unity. ThOD is the conservative form of COD. In most cases, ThOD of organic compounds may analytically be estimated by standard COD analysis. ThOD effectively accounts for the electrons involved in the biological redox processes.

Table 2.3: Typical stoichiometric parameters of ASM3.

Symbol	Characterization	Value	Units
f_{SI}	Production of S_I in hydrolysis	0	$gCOD_{SI}/gCOD_{XS}$
Y_{STO}	Aerobic yield of stored product per S_S	0.85	$gCOD_{XSTO}/gCOD_{SS}$
$Y_{STO,NO}$	Anoxic yield of stored product per S_S	0.80	$gCOD_{XSTO}/gCOD_{SS}$
Y_H	Aerobic yield of heterotrophic biomass	0.63	$gCOD_{XH}/gCOD_{XSTO}$
$Y_{H,NO}$	Anoxic yield of heterotrophic biomass	0.54	$gCOD_{XH}/gCOD_{XSTO}$
Y_A	Yield of autotrophic biomass per NO_3^--N	0.24	$gCOD_{XA}/gN_{SNOX}$
f_{XI}	Production of X_I in endogenous respiration	0.20	$gCOD_{XI}/gCOD_{XBM}$
$I_{N,SI}$	N content of S_I	0.01	$gN/gCOD_{SI}$
$I_{N,SS}$	N content of S_S	0.03	$gN/gCOD_{SS}$
$I_{N,XI}$	N content of X_I	0.02	$gN/gCOD_{XI}$
$I_{N,XS}$	N content of X_S	0.04	$gN/gCOD_{XS}$
$I_{N,BM}$	N content of biomass, X_H, X_A	0.07	$gN/g\ COD_{XBM}$
$I_{SS,XI}$	SS to COD ratio for X_I	0.75	$gSS/g\ COD_{XI}$
$I_{SS,XS}$	SS to COD ratio for X_S	0.75	$gSS/g\ COD_{XS}$
$I_{SS,BM}$	SS to COD ratio for biomass, X_H, X_A	0.90	$gSS/gCOD_{XBM}$

2.3.1.3 Process kinetics

In relation to removal of all soluble compounds, the kinetic expressions of ASM3 rely on switching functions, which are hyperbolic or saturation terms, Monod equations, S/(K+S). Similarly, the switching functions are affected for particulate compounds by the ratio of X_{STO}/X_H and X_S/X_H. All kinetic parameters of ASM3 regarding the heterotrophic organisms are given in Table 2.4. The units and typical values at 10 ˚C and 20 ˚C for these kinetic parameters are identified in Table 2.5.

2.3.2 Metabolic models

Being the first attempt to evaluate ASM3 using experimental data, Krishna and Van Loosdrecht (1999a) have observed that ASM3 failed to model two significant experimental observations: (i) the discontinuity in the growth rate of biomass observed experimentally in feast and famine phases and (ii) it required prediction of higher levels of internal storage polymers than measured to fit the oxygen consumption during feast and famine phases.

Guisasola et al. (2004), moreover, showed that for batch OUR data, ASM3 approach also causes severe practical identification problems that resulted in unrealistic and nonmechanistic parameter estimates which is the traditional way in model calibration. The major reason of this failure was the experimentally observed fact

that storage and growth occur simultaneously during the feast phase as opposed to the assumption of ASM3 that only storage occurs during the feast phase (Van Aalst-van Leeuwen et al., 1997; Krishna and Van Loosdrecht, 1999a).

Table 2.4: Kinetic rate expressions of ASM3.

j	Process	Process rate equation ρ_j, for all $\rho_j \geq 0$
1	Hydrolysis	$k_H \cdot \frac{X_S / X_H}{K_X + X_S / X_H} \cdot X_H$
2	Aerobic storage of COD	$k_{STO} \cdot \frac{S_O}{K_O + S_O} \cdot \frac{S_S}{K_S + S_S} \cdot X_H$
3	Anoxic storage of COD	$k_{STO} \cdot \eta_{NO} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO}}{K_{NO} + S_{NO}} \cdot \frac{S_S}{K_S + S_S} \cdot X_H$
4	Aerobic growth	$\mu_H \cdot \frac{S_O}{K_O + S_O} \cdot \frac{S_{NH}}{K_{NH} + S_{NH}} \cdot \frac{S_{HCO}}{K_{HCO} + S_{HCO}} \cdot \frac{X_{STO} / X_H}{K_{STO} + X_{STO} / X_H} \cdot X_H$
5	Anoxic growth (denitrification)	$\mu_H \cdot \eta_{NO} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO}}{K_{NO} + S_{NO}} \cdot \frac{S_{NH}}{K_{NH} + S_{NH}} \cdot \frac{S_{HCO}}{K_{HCO} + S_{HCO}} \cdot \frac{X_{STO} / X_H}{K_{STO} + X_{STO} / X_H} \cdot X_H$
6	Aerobic endogenous respiration	$b_{H,O2} \cdot \frac{S_O}{K_O + S_O} \cdot X_H$
7	Anoxic endogenous respiration	$b_{H,NO} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO}}{K_{NO} + S_{NO}} \cdot X_H$
8	Aerobic respiration of PHA	$b_{STO,O2} \cdot \frac{S_O}{K_O + S_O} \cdot X_{STO}$
9	Anoxic respiration of PHA	$b_{STO,NO} \cdot \frac{K_O}{K_O + S_O} \cdot \frac{S_{NO}}{K_{NO} + S_{NO}} \cdot X_{STO}$

Table 2.5: Typical values for kinetic parameters of ASM3.

Symbol	Characterization	Temperature		Units
		10 °C	20 °C	
k_H	Hydrolysis rate constant	2	3	$gCOD_{XS}/gCOD_{XH}.d^1$
K_X	Hydrolysis saturation constant	1	1	$gCOD_{XS}/gCOD_{XH}$
k_{STO}	Storage rate constant	2.5	5	$gCOD_{SS}/gCOD_{XH}/d$
η_{NO}	Anoxic reduction factor	0.6	0.6	-
K_{O2}	Saturation constant for S_{NO2}	0.2	0.2	gO_2/L
K_{NO}	Saturation constant for S_{NOX}	0.5	0.5	gNO_3^-N/L
K_S	Saturation constant for substrate S_S	2	2	$gCOD_{SS}/L$
K_{STO}	Saturation constant for X_{STO}	1	1	$gCOD_{XSTO}/g.COD_{XH}$
μ_H	Heterotrophic maximum growth rate of X_H	1	2	1/d
K_{NH4}	Saturation constant for ammonium, S_{NH4}	0.01	0.01	gN/L
K_{ALK}	Saturation constant for alkalinity for X_H	0.1	0.1	$gHCO_3^-/L$
$b_{H,O2}$	Aerobic endogenous respiration rate of X_H	0.1	0.2	1/d
$b_{H,NO}$	Anoxic endogenous respiration rate of X_H	0.05	0.1	1/d
$b_{STO,O2}$	Aerobic respiration rate for X_{STO}	0.1	0.2	1/d
$b_{STO,NO}$	Anoxic respiration rate for X_{STO}	0.05	0.1	1/d

The experiments carried out in activated sludge systems, operating under dynamic conditions indicate that although the storage of internal polymers is usually the main mechanism for the removal of readily biodegradable carbon sources, bacteria can grow on both external and internal substrate under dynamic conditions. It has been reported that bacteria use external substrate preferentially for cell growth (i.e. to optimize the growth rate) or for storage processes (i.e. to optimize the substrate uptake rate). This fact led to the formulation of the first simultaneous storage and growth model in order to interpret the experimental data better by Krishna and Van Loosdrecht (1999a). From a mechanistic modelling point of view, it becomes clear that ASM3 should be extended to account for simultaneous storage and growth. In addition to Krishna and Van Loosdrecht (1999a), several models have been proposed to improve the mechanistic modelling of simultaneous storage and growth processes in activated sludge systems.

Krishna and Van Loosdrecht (1999a) performed the first modelling study for the evaluation of ASM3, with measurement of the conversion of acetate, ammonium, oxygen, biomass and PHB at different temperatures in activated sludge. These measurements were compared with simplified version of ASM3 for the case of only aerobic heterotrophic conversions and acetate as the only substrate. All expressions and parameters were chosen according to the description of Gujer et al. (2000), except the conversion of particulate COD components to total suspended solid (TSS) and fraction of nitrogen in the biomass. First simulation results were not so successful in predicting the observed experimental results, because it gave too low acetate uptake rate, very high storage product turnover, low mixed liquor suspended solid (MLSS) concentrations and low OUR and growth rate for feast period. Therefore, in case of evaluation of the poor model prediction, the stoichiometric coefficients of simplified ASM3 were changed with earlier experiments of Van Aalst-van Leeuwen et al., (1997) carried out with pure culture of *Paracoccus pantotrophus sp*. while kinetic parameters remained the same. Results of new simulation did not supply much improvement in the acetate uptake rate, PHB formation and growth rate.

Finally, they tried to calibrate the kinetic coefficients for storage and growth (k_{STO} and μ) in order to remove the differences between the model prediction and experimental results. Alternative model structure, gave good accordance in OUR

while the growth rate still shows great difference. As a result, simplified ASM3 gives a reasonable description of the studied SBR process, but the discontinuity in growth rate between feast and famine period cannot be described. Therefore, the addition of the growth process on the primary carbon source to ASM3 was proposed (Krishna and Van Loosdrecht, 1999a). The modelling studies conducted with the assumption of simultaneous growth and storage on the external substrate has resulted in a better description of the system. Growth on storage polymer is determined by the consumption rate of the polymer, not by the growth rate potential of the cells. This means that in addition to the growth rate of biomass, the consumption rate of storage polymers should be used in mathematical models such as ASM3. Evidence so far indicates that the consumption rate on storage polymers should be described by a first order rate rather than saturation kinetics. The kinetic rate expressions used in this model is shown in Table 2.6.

Table 2.6: The kinetic rate expressions of metabolic model for aerobic conditions (Krishna and Van Loosdrecht, 1999a).

Process		Process rate Equation
1	Aerobic storage of COD	$k_{STO}.\frac{S_O}{K_O+S_O}.\frac{S_S}{K_S+S_S}.X_H$
2	Aerobic growth on X_{STO}	$\mu_H.\frac{S_O}{K_O+S_O}.\frac{K_S}{S_S+K_S}.\frac{S_{NH}}{K_{NH}+S_{NH}}.\frac{X_{STO}/X_H}{K_{STO}+X_{STO}/X_H}.X_H$
3	Aerobic endogenous respiration	$b_H.\frac{S_O}{K_O+S_O}.X_H$
4	Aerobic respiration of X_{STO}	$b_{STO}.\frac{S_O}{K_O+S_O}.X_{STO}$
5	Aerobic growth on acetate	$\mu_H.\frac{S_O}{K_O+S_O}.\frac{S_S}{K_S+S_S}.\frac{S_{NH}}{K_{NH}+S_{NH}}.X_H$

According to this metabolic model, in the presence of external substrate, the organisms have a choice to use the substrate for growth or storage processes. Many organisms subjected to feast-famine conditions maximize their substrate uptake rate while growing at a more or less balanced rate (Van Loosdrecht et al., 1997). The difference between actual substrate uptake rate and growth rate leads to substrate storage. This would mean that bacteria compete on substrate uptake rate rather than growth rate.

For simultaneous storage and growth, three yield factors have been introduced that are the yield for growth of biomass on substrate (Y_{SX}), the yield of storage on substrate (Y_{SP}) and the yield of growth on the stored substrate (Y_{PX}). If there is no simultaneous storage and growth, it is not necessary to define the yield for growth on external substrate. The schematic comparison of the ASM3 and metabolic model involving simultaneous growth and storage is illustrated in Figure 2.1.

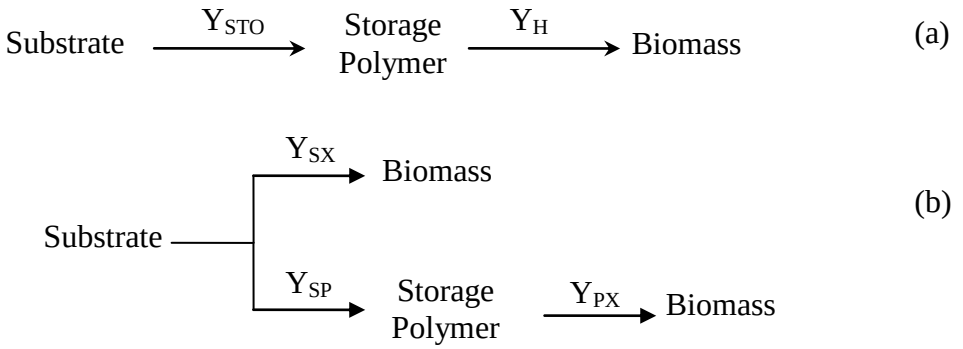


Figure 2.1: Metabolic route of biomass (a) in ASM3 and (b) metabolic model proposed Krishna and Van Loosdrecht (1999a).

Karahan-Gül et al. (2002) made a similar modification of ASM3 with the assumption of simultaneous growth and storage on external substrate. According to the proposed modified version, direct heterotrophic growth occurs on external substrate and after the depletion of external substrate; growth of biomass takes place on the internal storage product in the model. The proposed model consists of hydrolysis, endogenous respiration of biomass and respiration of storage products processes as described in ASM3.

Storage of readily biodegradable substrate and direct growth processes were described as simultaneous processes competing for substrate and electron acceptor. Both processes have reaction rates according to Monod kinetics where growth processes have ammonia nitrogen and bicarbonate limitations. Secondary growth processes are inhibited when primary substrate is present in the system as proposed by Krishna and Van Looshdrect (1999a). Oxygen uptake rate (OUR) experiments were carried out for acetate and results were compared with the OUR curve of the model proposed simulation. The results have provided strong indication that there was a need for considering direct growth on external substrate as a significant biological mechanism (Karahan-Gül et al., 2002).

The study carried out for the evaluation of the storage of slowly biodegradable substrate with starch as model substrate by Karahan et al. (2005) pointed out the rapid adsorption of the starch prior to hydrolysis as observed by the starch iodine test. In this test, while the starch was observed for 30 min in mixed liquor, the starch disappeared from the bulk liquid in 8 minutes in filtered sample. This result was confirmed by the prolonged pattern of the corresponding OUR curve with respect to depletion of the starch from bulk liquid. As a result of this observation, the insertion of the adsorption and hydrolysis processes to the simultaneous growth and storage model was suggested by Karahan et al. (2006). The general model for the simultaneous growth and storage of the readily and slowly biodegradable carbon sources is shown in Table 2.7.

Table 2.7: The model covering simultaneous growth and storage under aerobic conditions (Karahan et al., 2005).

Component / *Process*	S_O	S_S	X_S	X_{ads}	X_H	X_{STO}	*Process Rate*
Adsorption			-1	1			$k_{ads}.X_S$
Hydrolysis		1-f_{SI}		-1			$k_H \frac{X_S/X_H}{K_X + X_S/X_H} X_H$
Growth on S_S	$-\frac{1-Y_{H1}}{Y_{H1}}$	$-\frac{1}{Y_{H1}}$			1		$\mu_{H1}.\frac{S_S}{K_{S1}+S_S}.X_H$
Storage of S_S	$-(1-Y_{STO})$	-1				Y_{STO}	$k_{STO}.\frac{S_O}{K_O+S_O}.\frac{S_S}{K_{S2}+S_S}.X_H$
Growth on X_{STO}	$-\frac{1-Y_{H2}}{Y_{H2}}$				1	$-\frac{1}{Y_{H2}}$	$\mu_{H2}.\frac{K_{S1}}{K_{S1}+S_S}.\frac{X_{STO}/X_H}{K_{STO}+X_{STO}/X_H}X_H$
Endogenous Respiration	$(1-f_I)$				-1		$b_H.\frac{S_O}{K_O+S_O}.X_H$
Respiration of X_{STO}	-1					-1	$b_{STO}.\frac{S_O}{K_O+S_O}.X_{STO}$

As given in Table 2.7, for consumption with storage or direct growth, the slowly biodegradable COD is absorbed into biomass and then hydrolyzed to readily biodegradable substrate. The kinetic and stoichiometric parameters of simultaneous growth and storage model reported for the experiments carried out with different operating conditions are given in Table 2.8.

Table 2.8: Proposed values of kinetic and stoichiometric parameters.

Parameter	References				Units
	ASM3	Krishna and Van Loosdrecht 1999a	Karahan-Gül et al., 2003	Karahan et al., 2006	
Y_{STO}	0.85	0.73	0.80	0.91	gCOD/gCOD
Y_{H1}	-	0.50	0.65	0.79	gcellCOD/gCOD
Y_{H2}	0.63	0.65	0.75	0.84	gcellCOD/gCOD
k_{STO}	5	10	14	25	$gSS/gX_H.d$
K_{STO}	1	1	0.4	0.4	gX_{STO}/gX_H
K_{S1}	2	0.1	3	20	gCOD/L
K_{S2}	-	-	-	2	
μ_{H1}	-	3.5	4	3	1/d
μ_{H2}	2	2	3	4	1/d
b_H	0.20	0.2	0.24	0.10	1/d
b_{STO}	0.20	0.2	0.24	0.05	1/d
k_{ads}	-	-	-	360	1/d
k_H	-	-	-	30	1/d
K_X	-	-	-	0.05	gCOD/gCOD

2.4 Stoichiometry and Kinetic of Activated Sludge under Dynamic Conditions

As the cell has a variable specific activity level, any variation in process operating conditions or any modification of system will cause a deviation in microbial response. Basically, the rRNA level of cell is linked to the maximum growth rate (μ_{Hmax}) since it governs the PSS and μ_{Hmax} change with culture conditions.

On the other hand, the activity of enzyme, which is responsible for substrate transport, is the indication of substrate affinity of the cells. The affinity increases with the elevated activity of transport enzyme(s), which corresponds to low half saturation constant (K_S) values (Daigger and Grady, 1982; Kovarova-Kovar and Egli, 1998; Ferenci, 1999). It was observed by several authors that the specific substrate uptake rate varies with growth conditions, although; K_S also depends on the type of the substrate, substrate concentration and the metabolic state of the species responsible for substrate degradation (Kovárová-Kovar and Egli, 1998).

In this context, Ferenci (1999) showed that the high growth rate and low substrate concentration lead to a high affinity transport system (lower K_S). Moreover,

consortia of rapidly growing bacteria show higher affinity constant than slow growth species based on the competition principle (Stumm-Zollinger and Harris, 1971). Modifications in the population structure of the biomass may therefore cause variations in the affinity constant. Moreover, some chemical phenomenon, such as mass transfer resistance in large flocs, may affect the assessment of the affinity constant (Characklis 1978; Shieh 1980; Lau et al. 1984; Chu et al., 2003). In other words, although, the cell itself adapts to the environment, the population structure and floc size may affect the value of the affinity constant (Lavallee et al., 2005). Therefore, the formulation of affinity constant may be complex for different models.

The storage yield (Y_{STO}), is one of the most important parameters of the simultaneous storage and growth models, since it represents the stoichiometric amount of the substrate converted into storage products, which are then utilized for growth. The assessment of Y_{STO} is therefore crucial for the accurate estimation of the overall electron acceptor utilization and sludge production. As the Y_{STO} mainly depends on the carbon source, it is essential to evaluate the storage in relation to the type of the produced storage polymers. Consequently, the substrate uptake rate and consequently Y_{STO} rely on the external carbon source, which determines the type of storage polymers. It was also suggested that the storage rate (k_{STO}) is changing with environmental conditions (Hanada et al., 2001). As these stoichiometric and kinetic values highly depend on the environmental conditions, it is better to evaluate these parameters based on the operating parameters of activated sludge systems. The growth environment, namely operating conditions, controls the expression of the genetic capabilities of the organisms such as the levels of the enzymes, which vary with the growth environment. The type of storage polymer and operating conditions also determine the species of microorganisms present in activated sludge systems.

2.4.1 Storage polymers

The nature of the carbon and energy source affects the microbial response for a number of reasons. First, in order to biosynthesis of storage product or release of metabolic intermediates, the substrate must be transported rapidly into the cell. Furthermore, a storage response requires the rapidly degradation of substrate to the precursors of the storage polymers (i.e. pyruvate and acetyl-CoA). Therefore, storage does not generally occur when proteins or complex synthetic organic chemicals serve as the carbon source since the initial steps in their biodegradation are too slow to

cause a build up in the concentration of the precursors of the storage polymers. Thus, readily biodegradable carbon and energy sources are most likely to lead to storage or to release of metabolic intermediates during a transient response (Daigger and Grady, 1982).

According to Dawes (1985), there are 4 principal classes of storage polymers in microorganisms: lipids, carbohydrates, polyphosphates, and nitrogen reserve compounds. For lipid storage, the discussion will be focused mainly on polyhydroxyalkanoates (PHAs). Some organisms can accumulate more than one kind of storage polymers. For example, polyphosphate accumulating organisms (PAOs) that are responsible for phosphorus removal in BPR systems store glycogen, PHA, and polyphosphate. Dawes and Senior (1973) stated that environmental conditions and regulatory mechanisms influence the content of storage polymers accumulated. Stanier et al. (1976) stated that when a carbon substrate is metabolized via acetyl-CoA, the flow of carbon is mainly to PHA synthesis. However, if a carbon substrate is metabolized via pyruvate, glycogen storage is predominant.

On the metabolic level, glycogen storage is regulated by the adenylate energy charge. An excess of the carbon and energy source leads to an increase in the energy charge, which triggers glycogen biosynthesis. Control of PHB biosynthesis and degradation is primarily affected by the $NADH^{+}$/NAD ratio. Since this ratio is related to the energy charge, the levels of both glycogen and PHB are determined by the same mechanism.

2.4.1.1 Lipids (Polyhydroxyalkanoates, PHAs)

PHAs are polyesters of various hydroxyalkanoates that are synthesized by many Gram-positive and Gram-negative bacteria from at least 75 different genera (Reddy et al., 2003). Microbial production of PHA, both under aerobic and anaerobic conditions, has gained considerable interest because of the potential applications of these polyesters as biodegradable plastics (Brock and Madigan, 1991). PHAs can be thermoplastically formed and can be used as new plastics which have the advantage over polypropylene and polyethylene of being biodegradable. They can be transformed into sheets, fibres and hollow bodies like bottles and mugs (Schlegel, 1993). Besides being the only linear biodegradable polyester, they are also

biocompatible with numerous applications in medicine, pharmacy and packaging (Steinbüchel and Wiese, 1992).

More than 40 different hydroxyalkanoic acids (HAs) have been detected as constituents of PHA, but only few homopolyesters besides poly (3-hydroxybutyrate) (PHB) are available from bacteria (Steinbüchel and Wiese, 1992). The type of PHA components produced depends on the composition of substrate. Well known monomeric units of PHA in activated sludge are poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV). In addition to these two main polymers, the production of other PHA components such as 3-hydroxy-2-methylbutyrate (3H2MB) and 3-hydroxy-2-methylvalerate (3H2MV) may also be significant (Satoh et al., 1992). Since PHB is directly formed from the central metabolite Acetyl-CoA (Doi, 1990), it is considered as the most important storage polymer. PHB is present as granules enclosed by a membrane in the cytoplasm of the cells. The granules of PHB have typical diameters between 0.2 to 0.5 μm. Figure 2.2. depicts a general structure of PHAs.

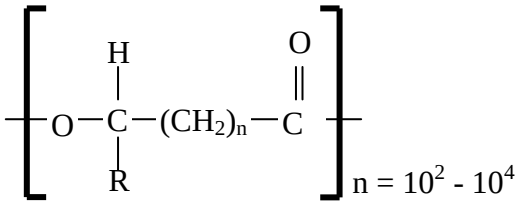


Figure 2.2: General Structure of PHA (R: methyl: PHB and R: ethyl: PHV).

The regulation of PHB synthesis is controlled at enzymatic level (Senior and Dawes, 1971) and mainly depends on the intracellular concentration of acetyl-CoA and free coenzyme A (CoA) (Haywood et al., 1988). The production of storage polymers is thought as serving a NADH overflow mechanism to control the redox state of heterotrophic cells during unbalanced growth conditions (Van Niel et al., 1995).

As illustrated in Figure 2.3, after bacteria take up acetate into cell, it is converted to acetyl-CoA and the produced acetyl-CoA is primarily used in the tricarboxylic acid (TCA) cycle to produce ATP, $NADH_2$ and to form biomass. When growth conditions are unbalanced, acetyl-CoA cannot enter TCA cycle to obtain energy for cells due to high concentrations of NADH (Doi, 1990). High concentrations of NADH inhibit citrate synthase, one of the key enzymes of TCA cycle, leading to an increase in the level of acetyl-CoA. For PHB synthesis, two molecules of acetyl-CoA are condensed

to form acetoacetyl-CoA and release a CoA. The enzyme 3-ketothiolase catalyzes this condensation reaction. The increase in the concentration of acetyl-CoA drives the equilibrium reaction of 3- ketothiolase into the direction of PHB synthesis. The acetoacetyl-CoA is reduced to (R)-3-hydroxybutyryl-CoA. This reaction is catalyzed by NADPH-dependent acetoacetyl-CoA reductase and (R)-3-hydroxybutyryl-CoA molecules synthesize PHB with the release of free CoA.

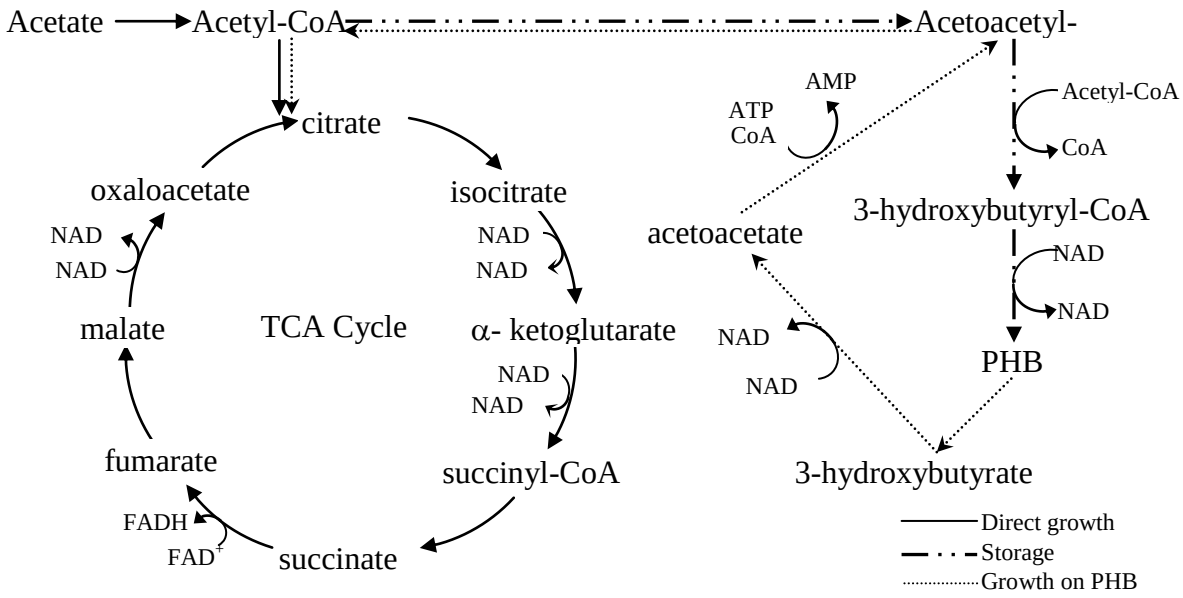


Figure 2.3: PHB production pathways in feast/famine conditions (adapted from Salehizadeh and Van Loosdrecht 2004).

Subsequently, PHB is accumulated in order to keep the concentration of intermediates at a balanced level in cells. It is likely that when the ratio acetyl-CoA/CoA is high, the cells will respond with the production of more anabolic enzymes, i.e. increase their growth rate. If the period of excess external substrate availability is long enough, the specific growth rate of the biomass will increase to its maximum and the PHB synthesis rate will slow down.

Depolymerization of PHB appears to be inhibited in the presence of a soluble substrate favouring rapid growth (Kessler and Witholt, 2001). However, certain observations seem to indicate that degradation of PHB occurs at the same time as its synthesis (Kessler and Witholt, 2001). When PHB is used by biomass while no external substrate is available, PHB is converted to D(-)-3-hydroxybutyrate by PHB depolymerase and then produced D(-)-3-hydroxybutyrate is converted to acetoacetate by D(-)-3-hydroxybutyrate dehydrogenase. Finally, acetoacetyl-CoA is formed by

acetoacetyl-CoA synthetase. Metabolic pathway involved in the synthesis and degradation of PHB require energy and reducing power. Although, the knowledge of the PHB degradation pathway is very limited, it is accepted that one of the steps in the biochemical pathway of PHB degradation must be the rate limiting.

2.4.1.2 Carbohydrates

Carbohydrate reserves include glycogen and glycogen-like materials. Glycogen is formed only when sugars are present in the influent, and it plays an essential role in bacterial metabolism (Preiss, 1984). A wide variety of organisms can accumulate glycogen and glycogen-like materials. The organisms capable of glycogen storage tend to prolong their viability during starvation periods by utilizing the storage reserve as happened in PHA production (Dawes and Senior, 1973; Preiss, 1984).

Glycogen is a branched polymer made up of glucose monomers connected by α-1,4 and α-1,6 glycosidic bonds. Glycogen granules are smaller and more dispersed than PHA granules (Prescott et al., 1990). Glycogen formation from glucose 6-phosphate, which is the intermediate product in biodegradation of glucose, is regulated by genetic expression and enzymatic activity (Preiss, 1996). The cell appears to regulate its metabolic capacity to convert the carbon substrate into glycogen in response to the substrate availability (Preiss, 1996). This regulation occurs at the level of gene expression through cyclic adenosine monophosphate (AMP) and guanosine tetraphosphate (ppGpp) (Preiss, 1996). Degradation of intracellular glycogen occurs when the exogenous carbon source is consumed. While a number of enzymes that are capable of hydrolyzing α-1,4 glucosidic bonds and α-1,6 linkages have been identified in bacteria, the precise details of glycogen degradation and its control are still incomplete. It is a general observation that the rate of glycogen utilization is significantly lower than its rate of synthesis (Dawes, 1992).

The general metabolism of carbohydrates and glycogen storage have been studied with taking into account the higher molecular weight sugars which are needed to exocellular hydrolysis before consumption by microorganism. In this framework, starch has been used as model substrate for the evaluation of the glycogen storage (San Pedro et al., 1994; Karahan et al., 2005). During the hydrolysis of starch, some fraction of hydrolysis product is consumed for oxidation and cell synthesis, and then remaining fraction is transformed into intracellular glucose (Matsuzawa and Mino,

1991). After the adsorption and hydrolization of starch, the glycogen may be produced by microorganisms related to the environmental conditions. The glycogen metabolism involving formation of the glucose from the adsorption of extracellular starch and hydrolysis of intracellular starch has been described as shown in Figure 2.4 (San Pedro et al., 1994).

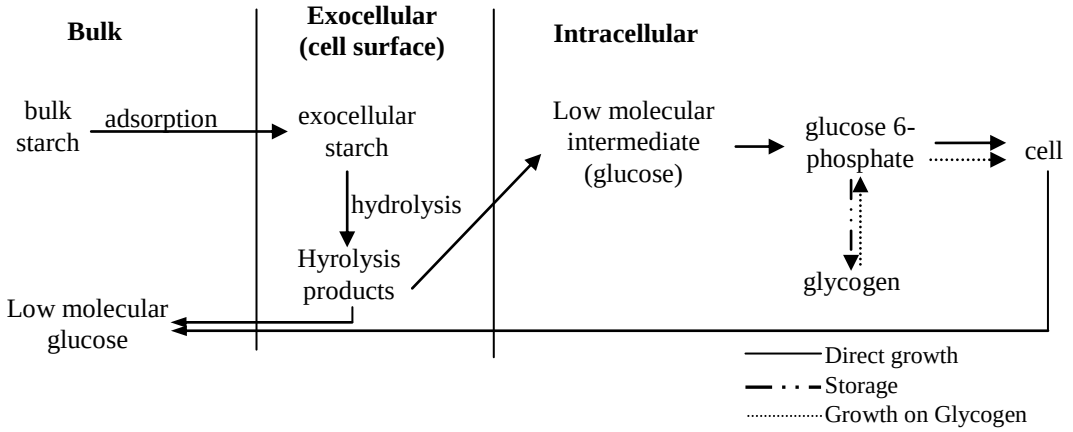


Figure 2.4: Schematic representation of glycogen metabolism (adapted from San Pedro et al., 1994).

As illustrated in Figure 2.4, low molecular weight intermediate glucose is produced from subsequent metabolism of starch via adsorption and hydrolysis. Then glucose-6-phosphate (G6P) is produced. The produced G6P can be converted into glycogen or directly used for biomass production via glycolysis. G6P is also used for catabolic reactions consuming oxygen. This reaction consumes ATP, but it acts to keep the glucose concentration low, promoting continuous transport of glucose into the cell through the plasma membrane transporters. After the depletion of primary substrate, glycogen is used for the synthesis of G6P.

2.4.1.3 Interaction between storage of PHA and carbohydrate

In their natural environments microorganisms often encounter situations when not a single carbon source, but mixtures of carbon and energy sources are present. Under such conditions, bacteria often utilize one carbon source preferentially, with the further carbon source(s) being consumed only, when the preferred one is exhausted. As already shown by Monod (1942), the preferred carbon source in general supports the best growth rate and/or growth yield, and the successive utilization of the substrates is often represented (Monod, 1942). In the environmental engineering point of view, the wastewater includes several substrates which have a different

degree of complexity. The internal metabolism of the cell is mainly based on the principal of the substrate removal process. Therefore, it is important to understand the interaction between the metabolic pathways used for the removal of different carbon sources.

In this context, the measurement of metabolic fluxes is important for establishing which pathways are active in a given physiological state and revealing the actual rates of relevant reactions. The use of stable-isotope tracers and the analysis of the distribution of labelled carbons in various intermediates, by both mass spectrometry and Nuclear magnetic resonance (NMR) spectroscopy, allow the characterization of specific metabolic routes (Santos et al., 1994; Neves et al., 2000; Xavier et al., 2000).

NMR is a spectroscopic technique based on the magnetic properties of atomic nuclei. Although the earlier metabolic studies have mainly relied on the measurements of the ^{1}H, ^{31}P, the use of ^{13}C-labeled substrates has become common in recent years because of the numerous advantages of the directly following the fate of carbon source during the bioconversion. In this respect, as the ^{13}C NMR reveals useful information on molecular structure and chemical reactions, NMR analyses have been applied to study a whole range of processes relevant to environmental engineering.

Solid state NMR provides powerful techniques for elucidating details of molecular dynamics and local conformation in solid materials as the use of concentrated cell suspensions or cell extracts is an elegant way to obtain intermediates in higher concentrations, especially for the study of anaerobic conversion processes. ^{13}C fractional enrichments and isotopomer distributions of biomass precursors from this part of central metabolism can be determined by NMR measurements of glycerol and the proteinogenic amino acids alanine, aspartate, glutamate, phenylalanine, serine, and threonine from biomass hydrolysates.

Glycolysis and the TCA cycle are the major catabolic pathways for many microorganisms. Therefore, the two amino acids, namely glutamate and alanine, are emphasized because of their direct relation with the intermediates of major catabolic pathways. Glutamate is highly abundant in most cell and is in rapid exchange with the TCA cycle intermediate, α-ketoglutarate (Berliner and Robitaille, 1999) although the labelling of alanine isotopologues reflects the labelling patterns of pyruvate derived from ^{13}C substrate metabolism such as glucose or more complex sugars.

Since the 1980s, NMR technology has been used to investigate various aspects of PHA including the monomer compositions, cellular content, copolymer analysis and metabolic pathways (Yan et al., 2000). In addition to the PHA, the use of ^{13}C-NMR in vivo is particularly suited to the study of glycogen cycling. Glycogen is not fully soluble in cellular extracts but can easily be detected in whole cells by NMR. In contrast to other high molecular- mass polymers, the molecular mobility of glycogen is high, a feature that favours its detection by NMR (Gaudet et al., 1992). Additionally, the opportunity to perform in vivo kinetic analysis of glycogen labelling enables the monitoring of its simultaneous synthesis and breakdown.

The interaction between the metabolic pathways of different substrates has mainly been studied for pure cultures. The carbon sources can either be co-metabolized or preferentially used by pure cultures. In latter case, the bacteria prefer most easily accessible carbon source which allow fastest growth. The researches with bacteria and higher organisms have revealed that selective carbon-source utilization is common and that glucose is the preferred carbon source in many model organisms that have been studied. Moreover, the presence of glucose often prevents the use of other, secondary, carbon sources (Gorke and Stülke, 2008). Although, carbon source utilization by *Escherichia coli* and *B. subtilis* is typically characterized by the sequential utilization of the carbon sources present in a substrate mixture with glucose being the preferred substrate (Lin, 1996), the co-utilization of a mixture of acetate and glucose by *Corynebacterium glutamicum* has been reported (Wendisch et al., 2000). When the growth of *Corynebacterium glutamicum* on mixtures of the carbon sources was examined, the different rates on the removal of glucose and acetate than on either substrate alone were noticed. The organism showed nondiauxic growth on media containing acetate-glucose mixtures and simultaneously metabolized these substrates. Compared to those for growth on acetate or glucose alone, the consumption rates of the individual substrates were reduced during acetate-glucose co-metabolism, resulting in similar total carbon consumption rates for the three conditions (Wendisch et al., 2000). This result indicates that either the uptake of acetate and of glucose or the in vivo carbon fluxes from glucose to acetyl-CoA and from acetate to acetyl-CoA are directly or indirectly regulated by the carbon source in the growth medium. In contrast to *C. glutamicum, Azotobacter vinelandii* preferentially uses acetate when grown on glucose-acetate mixtures, which

is due to acetate-dependent inhibition of glucose uptake and glycolysis (George et al., 1985; Tauchert et al., 1990). On the other hand, *Escherichia coli* preferentially uses glucose when fed with mixture of glucose and acetate due to carbon catabolite repression of the acetate-activating acetyl-CoA synthetase and probably also of the glyoxylate cycle enzymes isocitrate lyase and malate synthase in the presence of glucose (Brown et al., 1977). The metabolism of *Escherichia coli* during growth on glucose-acetate mixtures is very similar in the first phase to growth on glucose as a sole carbon source and in the second phase to growth on acetate as a sole carbon source (Walsh and Koshland, 1985). In another study carried out with *Escherichia coli* on a mixture of glucose and organic acids like lactate, pyruvate and acetate, a lower acetate consumption rate was observed in the presence of the glucose, even if both substrates were consumed at the same time (Doshi and Venkatesh, 1998).

The detectability of polymers by in vivo NMR is an important issue because they can exist in highly immobilized structures that give rise to severe line broadening. In activated sludge, 40% of the glycogen pool was invisible although the PHAs were fully detected (Pereira et al., 1996). The studies on the behaviour of mixed culture in the presence of the multiple have been mostly studied under anaerobic conditions, in the frame of enhanced P removal processes. The studies have mainly focused on the PHA utilization under anaerobic conditions and conversion of PHA to glycogen under subsequent aerobic conditions (Mino et al., 1995; Pereira et al., 1996; Serafim, 2002). The relative amount of PHA and glycogen formed in mixed cultures under alternating anaerobic/aerobic conditions has been found to depend on the competitions between Phosphorus Accumulating Organisms (PAOs) and Glycogen Accumulating Organisms (GAOs) which use acetic acid and glucose respectively (Mino et al., 1995). It has indicated that the metabolism of this organism is quite different from those that are active in EBPR activated sludge, even though the P uptake rates of these organisms are similar (Santos et al., 1999).

On the contrary, under aerobic conditions, the effect of dynamic conditions on storage mechanisms has been studied in great detail by using single substrates whereas less attention has been given to the effect of the interaction of different substrates on the removal mechanisms of each other. In a study carried out under aerobic conditions with a mixture of similar substrates (i.e. a mixture of acetic, lactic and propionic acid), a strong decrease of the removal rates of acetic and lactic acid

was observed in the presence of another substrate, than when using each substrate as the only carbon source (Dionisi et al., 2004). This strong interaction among the different substrates suggests that they are removed by the same microorganisms that utilize same or interconnected pathways.

Less clear evidences are available when dealing with mixtures of more different substrates, like volatile fatty acids and carbohydrates. In the study carried out with a mixture of glucose and acetate, Carta et al. (2001) has reported that there are no differences in the uptake of acetate and glucose compared to the experiments performed with the single substrate. In addition to the substrate uptake rates, the degradation kinetics and rates of the storage compounds were also reported as the same as for the systems in which only one compound was stored in the activated sludge. In another study, the individual removal of acetate and starch (and respective storage of PHA and glycogen), was observed at slightly lower rates as compared to single substrate (Karahan et al., 2008).

The only NMR study regarding the aerobic metabolism of mixed substrate has been performed by Dionisi et al. (2004). In this study, the PHB storage was observed during glutamic acid removal as the only carbon source by activated sludge cultivated under aerobic dynamic conditions. The study has indicated that the presence of the storage simultaneously with direct growth on primary substrate as the one of the metabolic pathway involved in glutamic acid removal; even though an amount of storage product from glutamic acid was not as high as with other substrates, like acetate.

2.4.2 Effect of SRT on storage kinetics

As the average growth rate of the biomass is mainly controlled by the sludge retention time (SRT), SRT has been used for the determination of the mean microbial life-time, and hence microbial population in activated sludge systems (Frigon, 2006). Although, ASM3 suggested the default value of storage yield as 0.85 gCOD/ gCOD, extensive experimental studies showed that storage yield ranges from 0.32 to 0.79 gCOD/gCOD. Storage yield values for different substrates and related sludge retention times (SRT) found in several studies are given in Table 2.9. As seen from the Table 2.9, different yield values were reported by different authors for the same substrates. The storage yield for storage of PHB from acetate was found as 0.38

gCOD/gCOD at the SRT of 3.8 days (Beun et al., 2000) and 0.47–0.59 at the SRT of 6.9–10.4 days (Martins et al., 2003).

Table 2.9: Storage yield (Y_{STO}) values calculated at different SRTs.

Reference	Growth conditions of Activated Sludge		Carbon Source	Y_{STO} (gCOD/gCOD)
	SRT (days)	Feed composition (Ratio, COD based)		
Beun *et al.* (2000)	3.8	Acetate	Acetate	0.69
Beun *et al.* (2002)	2-20	Acetate	Acetate	0.68
Carta *et al.* (2001)	6.1	Acetate + glucose (1:1)	Acetate +glucose	0.6-07
Dionisi *et al.* (2004a)	1	Acetate + Lactate + propionate (2:2:1)	Acetate	0.49
			Propionate	0.38
			Lactate	0.32
Dircks et al. (2001)	7.7	Glucose	Glucose	0.91
Goel *et al.* (1998)	10	Acetate + Glucose + Peptone + Yeast Extract (2:1:1:1)	Glucose	0.68
			Acetate	0.45
			Starch	0.36
Karahan *et al.,* 2005	10	Starch	Starch	0.91
Krishna and Van Loosdrecht (1999a)	2.5	Acetate	Acetate	0.73
Martins *et al.* (2003)	7-10	Acetate	Acetate	0.47-0.59
Van Aalst-van Leeuwen *et al*. (1997)	0.5	Acetate	Acetate	0.73

For the storage of starch as glycogen, the storage yield was calculated as 0.36 gCOD/gCOD (Goel et al., 1998) at the SRT of 10 days, whereas same yield was determined as 0.91 gCOD/gCOD experimentally for soluble starch at the same SRT (Karahan et al., 2005). The storage yield for glycogen from glucose was found as 0.96 g COD/g COD (Chudoba et al., 1985). This difference in the storage yield of different type of substrates is explained by the less energy requirement glycogen storage than the PHB storage. Another explanation was that the high capacity-low affinity enzymes utilized for glucose could be substituted by low capacity-high affinity enzymes used for acetate degradation during the same experiment (Grady et al., 1996; Kovarova-Kovar and Egli, 1998).

Alternatively, it was found that activated sludge with an SRT of 3 days had better PHA production capability than sludge with an SRT of 10 days (Chua et al., 2003).

This observation was explained with the selection of microbial community having higher PHA production capacity at shorter SRTs than longer SRTs. On the contrary, Van Aalst-van Leeuwen et al. (1997) observed that faster growing organisms accumulated less PHB. In addition to this observation, Majone et al. (2007) showed that the overall transient response (i.e. maximum specific substrate removal rate) increased as SRT decreased in their pure culture study. Same statement has been made by Van Loosdrecht and Heijnen (2002) that the shorter the SRT, the higher the growth rate and the less substrate converted into storage polymers.

The low substrate uptake rate and PHA production rate have been observed in the SBR fed with acetate at SRT of 1 day, and then the SBR operated at SRT of 10 days although high organic loading was applied at low SRT (Lemos et al., 2008). The authors have concluded that the biomass was dominated by species which have high specific growth rates at low SRTs, as the growth yield was higher despite of lower uptake rates in low SRT.

The faster substrate uptake rate ($-q_S$) has been usually interpreted as indirect evidence of more relevant presence of a storage response in activated sludge process (Van Loosdrecht et al., 1997). The uptake rate is limited by the substrate concentration. Although a linear relation exists between substrate uptake and biomass growth in the systems where only biomass growth is observed, more complicated relations are present between the substrate uptake rate, biomass growth and PHB production. Studies on the storage phenomenon with pure cultures at low SRT (i.e. high growth rate) showed that storage is dependent on the growth rate of the culture (Van Aalst-van Leeuwen et al., 1997). In other words, the accumulation rate of storage products was linearly correlated to the difference between the maximum substrate uptake rate and the substrate uptake rate required for growth. When the culture is operated at a growth rate close to its maximum substrate uptake rate, storage is observed to be negligible. In this range, storage rate changes slightly with SRT while growth rate is strongly affected by variation of SRT (Van Loosdrecht and Heijnen, 2002). In addition, in the mixed culture, specific PHB production rate in the feast period was determined to be almost constant for different SRTs. Higher substrate uptake rate was observed in the culture acclimated at the SRT of 3.8 days, than the culture acclimated at the SRT of 9.5 and 19.1 days, which were essentially the same (Beun et al., 2000). Briefly, experiments showed that while substrate uptake rate ($-q_S$)

decreased linearly, storage compound production rate (q_P) linearly increased in the feast period when extra cellular substrate is available. The fraction of substrate removed to storage polymer produced ($q_P/-q_S$) is used as an indication of which fraction of the substrate is stored. Substrate uptake rates were found as 0.54-0.31 g COD/g COD.h for different SRTs when acetate was used as the carbon source (Beun et al., 2000) and 1.75-1.53 gCOD/gCOD.h for different SRTs when glucose was used as substrate (Dirks et al., 2000).

2.4.3 Effect of feeding pattern on storage kinetics

The feeding pattern affects occurrence of storage and release of metabolic intermediates through the selection of microbial species and the regulation of enzyme synthesis (Daigger and Grady, 1982). Those organisms which are capable of storing and releasing metabolic intermediates will have a competitive advantage in an environment where the feeding pattern is periodic, and thus they will be selected.

When feeding is continuous and stable, however, no competitive advantage is associated with the ability to form storage products or the release of metabolic intermediates. Likewise, periodic feeding will encourage the synthesis of enzymes required for biosynthesis of storage products and release of metabolic intermediates. Consequently, storage and the release of metabolic intermediates are more likely to occur during the transient response of a culture which was selected and maintained using periodic feeding than with a culture which was selected and maintained using stable continuous feeding. (Majone et al., 1996; Van Looshdrect et al., 1997).

For the evaluation of the contribution of the storage to substrate removal, two parallel continuously stirred reactor (CSTR) were operated at the SRT of 3 days with the continuously and intermittently acetate addition by Majone et al. (1996). Although different feeding patterns resulted with the different dominant microbial groups namely filamentous bacteria in continuously fed CSTR and floc forming bacteria in intermittently fed (2 min) CSTR, significant amount of PHB storage was observed in both CSTR. The storage capability of the filamentous bacteria even under intermittent feeding was confirmed with the following study of the same group (Beccari et al., 1998).The observation of the different population dynamics under same operating conditions in these two studies was explained with the important role of the initial inoculum on the microbial composition.

Several pure culture studies on relevant microorganisms confirmed that more or less strong dynamic conditions affect the relevance of storage, even if all other culturing conditions are the same, e.g. organic loading rate and culture residence time. A short-term dynamic effect has been clearly observed by applying a substrate spike to cultures previously grown under balanced conditions. In most cases, microorganisms were shown to be able to immediately increase their substrate uptake rate and the quick storage of substrate was considered as the reason for the observed phenomenon (even though not always measured). Clearly, the effect of transient conditions on the physiological state and related transient response of single microorganisms can be different from species to species (Majone and Tandoi, 2002) and they can affect the microbial population dynamics in the activated sludge. A similar short-term effect was also observed for mixed cultures; Van Loosdrecht et al. (1997) tested pulse feeding of acetate on the system previously operated at steady state with continuous feeding and observed immediate PHA formation. Similarly, Martins et al. (2004) has operated a continuously fed SBR under anoxic conditions and observed PHA storage when pulse feeding was applied in a single cycle. Other pure culture studies have clearly shown that the role of storage (yield and rate) is also depending on operating conditions (organic load rate and culture residence time), even with well-adapted microorganism (Dionisi et al., 2005; Majone et al., 2007). The activated sludge studies have typically been performed for comparing dynamic response of sludge acclimated under continuous feeding (bulking sludge) and intermittent feeding (Majone et al., 1996; Martins et al., 2003). These different cultivation conditions can affect both microbial population and physiological state of the sludge.

The differences between pulse and continuously fed systems have been cleared out by Pot et al. (1996). According to this study, all substrates were transformed to biomass at a constant rate and biopolymers were not formed under steady-state conditions. When the steady state continuous cultures were subjected to substrate pulse, the organism took up the substrate at a high rate. A direct increase in growth rate was not observed due to the change in substrate uptake rate. Polymer was formed by the conversion of excess substrate to storage polymer. As a result of this observation, the different mechanisms have been suggested as important for pulse and continuously fed systems. The observed rates and yields for the biomass acclimated under different feeding length are shown in Table 2.10.

Table 2.10: The rates and yields obtained from different feeding patterns.

References	SRT (days)	Feeding/ Cycle Length (min/min)	mgCOD/gCOD.h		Y_{STO} (gCOD/gCOD)
			$-q_S$	q_P	
Beccari et al., 1998	3	2/360	740-920	510-610	0.69
Beun et al., 2000	3.8	3/240	538	210	0.38
	9.5	3/240	362	210	0.57
	19.8	3/240	311	179	0.56
Krishna and Van Loosdrecht,1999a	2.5	55/240	203	112	0.55
Majone et al.,1996	3	Continuous	200-260	n.r.	0.35
		2/240	700-800	420-640	0.6-0.8
Beun et al., 2002	4	3/240	715	421	0.56
Martins et al., 2003	6.9-10.4	3-90/240	361-655	156-327	0.47-0.59
Martins et al., 2010	10	3/240	529	371	-
		50/240	79	11	-

As reported in Table 2.10, intermittently fed systems typically exhibited faster substrate uptake and higher observed yields than continuously fed systems. This has usually been explained by a more relevant presence of those microorganisms that are most able to store substrates quickly during the imposed transients and subsequently reuse them for growth, the more they have a competitive advantage and the resulting enriched activated sludge will have a higher storage response (Majone et al., 1996). In the pure culture study performed under periodic feeding conditions, the decrease in storage response was observed when the feeding period changed from pulse through the long feeding period, nearly continuous addition of carbon source, mainly due to the limited substrate concentration (Van den Eynde et al., 1984, Aulenta et al., 2003).

On the other hand, Majone et al. (1996) and Van Loosdrecht et al. (1997) have shown that the storage response is the main mechanism for both intermittently and continuously fed sludge. Also, the PHB accumulation has been observed in carbon limited systems (Van Aalst-van Leeuwen et al., 1997; Krishna and Loosdrecht, 1999a). The production of PHB even under carbon limited chemostat could indicate that the formation of PHB is an intrinsic part of microbial growth physiology (Van Aalst-van Leeuwen et al., 1997).

Moreover, although they observed a rapid decrease in growth rate with the increasing length of the feeding period, mainly due to a limited substrate concentration, the constant biomass specific enzyme level has been found as constant and independent of the length of acetate feeding period. According to this observation, Van Loosdrecht and Heijnen (2002) have suggested that cells grown under long feed periods have the same kinetic characteristics as when grown at pulse feeding.

In recent study, the effect of feeding length on the glycogen storage was evaluated by Martins et al. (2010). The completely adsorption of starch was observed by the iodine test (blue spots on the floc surface and not in the bulk liquid, indicating the formation of the starch iodine complex at floc level). The very high storage capacity with starch was observed as almost 80% similar to the result of 75% storage obtained by Karahan et al. (2005). Comparison of the storage capacity in respect to the feeding length showed that storage polymers are an intrinsic part of microbial physiology and ecology of activated sludge processes even in the long fed systems in which the specific substrate uptake rate reflects mainly the feeding pattern of the system due to the substrate limitation (Martins et al., 2010). Although low soluble substrate concentration was expected to lead the bulking sludge, there was no bulking observed. This observation was explained with the uniformly distribution of the hydrolysis products inside the floc. When substrate is taken up at low concentrations form the bulk solution this would lead to gradients over the floc giving advantage to filamentous organisms which extend outside the floc.

2.4.4 Process design

The SBR technology was developed to gain control over structure and functions of a heterogeneously composed microbial community in a multipurpose bioreactor exposed to a varying feeding regime (Irvine et al., 1971). SBR is increasingly being the preferred wastewater treatment reactor system for improved nutrient removal. If conditions are controlled appropriately, removal of all nutrients, including nitrogen, phosphorus, and organic carbon, can be achieved in a single reactor. The most distinct difference between the SBR and a conventional plug-flow activated sludge system is the dynamic and periodic nature of the sequencing batch reactor. SBR is ideal for selection of a robust population with an elevated ability for PHA production, because the biomass grows under transient conditions (Irvine et al., 1971). Alleman and Irvine (1980) recognized that operating strategies intended to

promote cellular storage, such as that offered by SBR, may reduce or eliminate the costly necessity for a supplemental carbon addition that is often required when treating wastewaters with a low carbon: nitrogen (C:N) ratio.

This kind of reactor is easy to control and highly flexible, allowing for a quick modification of the process conditions (i.e., the period of feeding and the cycle time). Transient conditions are created naturally in SBRs where steps of traditional activated sludge plants are replaced by periodic time sequences in a single reactor (Wilderer et al., 2001). Typical cycles of SBR include feed-reaction-settling-draw, where the reaction time can be further split into different phases (e.g. anoxic and aerobic). Moreover, a periodic process has additional degrees of freedom such as the length of the cycle and the length of the feed period that can be easily controlled at the same SRT.

2.4.5 Tools for the modelling of the dynamic behaviour in activated sludge

2.4.5.1 Oxygen uptake rate (OUR)

The respirogram and oxygen uptake rate profiles can be used for the selection of the best model and accurate estimation of model parameters. In this framework, the OUR profile together with the related chemical analysis results as carbon source consumption and microbial product formation are useful to collect necessary information for modelling (Insel et al., 2003). The oxygen uptake rate profile provides reliable information on the stoichiometry and kinetics in terms of both biomass activity and the biodegradability of carbon sources (Ekama et al., 1986; Spanjers and Vanrolleghem, 1995). The use of the OUR for the modelling of aerobic carbon oxidation in the context of ASM1 has been studied extensively in the literature (Kappeler and Gujer, 1992; Vanrolleghem et al., 1995).

It has been shown that the biomass yield, the maximum biomass growth rate and the substrate affinity constant could be obtained from the OUR profile resulting from a single batch test (Vanrolleghem et al., 1999). Current achievements with experimental methods based on respirometry and the oxygen uptake rate enabled a more consistent assessment of the kinetic coefficients involved, namely the maximum specific heterotrophic growth rate, μ_{Hmax} and the half saturation constant for heterotrophic growth, K_S for different substrates, domestic sewage and various industrial wastewaters (Insel et al., 2003). Because one of the goals is to assess the

biological characteristics of the wastewater, it is important that the activated sludge is respiring in the absence of the exogenous substrate; also called the endogenous respiration (Spanjers and Klapwijk, 1990). The time period to aerate the sludge before it reaches the endogenous state depends on the loading regime of the plant and the spot where the sludge was sampled. For detection of the endogenous state of the bacteria, aeration of the sludge was suggested for a period of 2 h before the feeding of carbon source (Spanjers and Klapwijk, 1990).

2.4.5.2 Model calibration and parameter identifiability

The examination of the dynamic behaviour in the activated sludge requires the determination of the optimal values of stoichiometric and kinetic parameters. In this context, the model calibration serves as a useful and reliable tool. The model calibration mainly relies on selection of the appropriate model representing the dynamic behaviour of reactor in selected operating conditions and fitting the model simulation with average experimental data. During model calibration, it is essential to fit the mass balance of biomass and sludge production to actual measurements in addition to the substrate consumption and related microbial product formation profiles (Insel et al., 2003). In this way, the stoichiometric and kinetic parameters of metabolic processes taking place in activated sludge can be estimated. Also, the overall steady state simulation of the model can be carried out using the determined default parameters for specific operating conditions.

In order to support environmental scientists in finding an "adequate" model for the system they are investigating; a computer program is necessary that allows its users to perform simulations for different models, to assess the identifiability and to estimate the values of model parameters (using measured data), and to estimate prediction uncertainty (Reichert et al., 1995). For this purpose, most widely used computer program is the AQUASIM which was developed by the Swiss Federal Institute for Environmental Science and Technology (Reichert et al., 1994). AQUASIM allows the stoichiometry and kinetic equations to be defined in relation to biological conversions, hydraulic effects, and reactor configuration and provides methods of sensitivity analysis, parameter estimation and uncertainty analysis in addition to simulation.

The identifiability is important, as it is an indication of the complication of activated sludge model (ASM) to apply automatic mathematical calibration technique. Identifiability is defined as the ability to obtain a unique parameter set that is able to describe the behaviour of a system. Typically, such experiments aim the identification of a few model parameters or components of the full ASM and the data analysis is therefore often carried out via reduced sub-models based on ASM. Indeed, a major problem encountered in calibration of ASM is the lack of identifiability of the model parameters. Identifiability is the ability to obtain a unique combination of parameters describing system behaviour. A distinction should be made between theoretical and practical identifiability.

Theoretical identifiability is a property of the model structure, and relates to the question whether it is possible to obtain unique parameter values for a given model structure considering certain selected outputs, and assuming ideal measurements. The practical identifiability on the contrary not only depends on the model structure, but is also related to the experimental conditions together with the quality and quantity of the measurements.

2.4.5.3 Practical identifiability analysis

The measured kinetics of a bacterial culture degrading a single organic compound as sole carbon source in a batch reactor depend on the identifiability of the parameters as well as the sludge history (Grady et al., 1996). Although, the sludge history is main indicator of the dominant species and their enzyme systems as well as the physiological state, the importance of the parameter identifiability arises from the ability of the mathematical routine used for parameter estimation to uniquely estimate the values of the individual parameters (Grady et al., 1996).

The experimental approach should be essentially taken into consideration for reflecting the exact sludge history and for availability in parameter identifiability. Although for the operation and optimization of activated sludge plant, either the model parameters are adjusted with model calibration (Koch et al., 2000) or the sensitivities of these parameters are analyzed for the interpretation of the parameter identifiability problems (Reichert et al., 1995). It would not be effective for the model consisting of large amount of stoichiometric and kinetic coefficients such as ASMs. For large models, Brun et al. (2001) has proposed an approach for parameter

identifiability as well as the evaluation of the interdependencies of the parameters. Parameter identifiability refers to the ability of the mathematical routine used for parameter estimation to uniquely estimate the values of the individual parameters.

The parameter identifiability is important since it allows obtaining some insight about adequate parameter values and comparing the analysis of same model to different data sets for complex systems. In literature, two aspects have been used for the parameter identifiability based on the model structure only (structural identifiability; Dochain et al., 1995) or on the type and quality of available data (practical identifiability; Vanrolleghem et al., 1995). As the large models like ASMs consist of numerous kinetic and stoichiometric parameters which depend on the sludge history, the primary aim of the parameter identifiability analysis is to find physically reasonable parameter values that describe the data adequately rather than the determination of "true parameter values". For this purpose, the practical identifiability serves as a useful tool for the determination of identifiable parameters (Dochain et al., 1995; Brun et al., 2001). Practical identifiability of a model structure is important as it tells which parameter combinations can be estimated under given measurement accuracy and quantity. In this way, one can improve the reliability and accuracy of the parameter estimation (Dochain and Vanrolleghem, 2001).

The approach proposed by Brun et al. (2001) has combined the sensitivity measures and determining the linear dependency of the parameters in terms of collinearity index (γ_K). In this way, this approach allowed to determine the degree of identifiability of parameter subsets and to compare the collinearity indices of parameters. In this approach if a parameter subset fits two conditions, it is accepted as identifiable. First, the model output has to be sufficiently sensitive to individual changes of each parameter in a parameter subset. Second, changes in the model output due to changes in single parameters may not be approximately cancelled by appropriate changes in other parameters in a parameter subset. The first condition is addressed by the sensitivity measure which is calculated for every parameter separately, the second by the collinearity index; which is calculated for arbitrary parameter subsets.

The sensitivity analysis, which is applied as a first step of the identifiability analysis, has been used for the determination of the change in the model output with respect to the model parameters. With sensitivity analyses, it is possible to detect near-linear

dependencies and insensitive parameters fairly efficiently (Reichter et al., 1995). A low sensitivity indicates that it is difficult to assign a unique value to the given parameter. Further, the similarity in the shapes of the sensitivity profiles of a measured variable to different parameters indicates the estimates of these parameters are likely correlated (Vanrolleghem et al., 1995). After the sensitivity analysis have been made for a model output, which is fit to experimental data, the following steps of the parameter identifiability have been described as the analysis of parameters on an individual basis with the sensitivity measures of δ_j^{msqr} and δ_j^{mabs} and then analysis of parameter subsets with respect to the collinearity indices (Brun et al., 2001):

$$\delta_j^{msqr} = \sqrt{\frac{1}{n}\sum_{i=1}^{n} s_{ij}^2} \qquad \textbf{(2.3)}$$

$$\delta_j^{mabs} = \frac{1}{n}\sum_{i=1}^{n} \left| s_{ij} \right| \qquad \textbf{(2.4)}$$

The parameter importance can be determined by ranking the parameters by one of the δ measures in decreasing order. In the context of weighted least squares estimation of parameter subsets, δjmsqr is suggested as best suited to serve as a ranking criterion. After determination of the suited parameter subsets for identifiability analysis with the sensitivity measures, the collinearity index (γ_K), which measures the extent of linear dependency between the sensitivity functions, can be defined by the following equation:

$$\gamma_K = \frac{1}{\min_{\|\beta\|=1} \left\| \tilde{S}_K \beta \right\|} = \frac{1}{\sqrt{\min \tilde{\lambda}_K}} \qquad \textbf{(2.5)}$$

where min λ_K is the minimum eigenvalue of the normalized subset matrix. The γ_K of a parameter subset has been used for determination of the sensitivity of an observed model output to small changes of all parameters in subset on an individual basis. While important parameters for a subset should be selected according to the parameter importance ranking, critical values for γ_K have been suggested as in the range of 5-20 by Brun et al. (2001). According to Belsley (1991), collinearity problems going along with condition numbers below 10 are rare. On the other hand, the values above 100 have been reported as the reason of the severe parameter identifiability problems. A collinearity index of 20 means that a change of the

calculated results caused by a shift of a parameter can be compensated to 5% by appropriate changes in the other parameters in a subset.

2.5 Population Dynamics in Activated Sludge Systems

When dealing with mixed cultures, the type and extent of biomass response to the transient conditions are determined by both on microbial composition of the consortium and on the physiological state of any microorganisms in the consortium, which is also affected by the operating conditions. Moreover, in highly dynamic systems such as activated sludge systems there are several and complex synergetic and antagonistic relationships among bacteria and between other organisms; and other factors play a key role as well. There is a growing interest to understand the population dynamics in activated sludge with respect to global metabolism of biomass.

Chudoba et al. (1973; 1985) proposed that all the bacteria in activated sludge systems could be classified into two groups as floc formers and filamentous bacteria. Thus, microbial populations dominated by filaments would be more commonly associated with completely mixed systems, but plug flow systems and SBRs would rarely bulk (Seviour and Nielsen, 2010). In bulking sludge, excessive levels of filaments interfere with solids settling and compaction (Grady and Daigger, 1999), resulting in solids inventory problems and high suspended solids concentrations in the effluent. For an efficient and economical treatment of wastewater, the settleability of activated sludge is of great importance and the poor settleability is often due to an imbalance between floc forming and filamentous bacteria, preventing the formation of well settling sludge flocs (Jenkins et al., 2004). Filamentous species, for which the main focus so far has been related to their inducement of filamentous bulking by overgrowth in wastewater treatment plants, are important members of the bacterial consortium (Jenkins et al. 2004). Thus, it is important to understand the competition between floc-formers and filaments and determine the effect of different factors on the selection of one type of microorganism over another in activated sludge systems. The growth conditions of the filamentous bacteria in activated sludge have mainly been studied regarding to the kinetic selection theory and storage phenomena.

2.5.1 Kinetic selection theory

The kinetic selection theory (KST) hypothesized by Chudoba et al. (1973) is the most widely used theory for modelling, selection and competition between filaments and floc formers. According to this theory filaments and floc-formers have different kinetic constants, K_S and μ_{Hmax} for a soluble substrate; and floc formers are generally thought to have high K_S and μ_{Hmax} for soluble substrate. Therefore, when the substrate concentration is low, filamentous bacteria have a higher substrate uptake rate than floc formers, and thereby win the competition for substrate (Chudoba et al. 1985).

A practical consequence of the theory is the use of smaller reactor before the main aeration basin. This smaller reactor has a higher process loading factor (because of a lower hydraulic retention time), and therefore it serves to select for floc-forming bacteria. The design and operation of such selectors has recently been the subject of intensive research based on the kinetic selection of filaments and floc formers (Wilderer et al. 2001; Martins et al. 2003). The KST has been used in a variety of models for predicting the outcome of the competition between the two organism types. For example, a dual substrate competition model was developed by Lau et al. (1984) for a floc former (*Citrobacter*) and the filament *Sphaerotilus*. The model incorporated the effect of carbonaceous substrate and dissolved oxygen diffusion to the floc. Van Niekerk et al. (1987) developed a competitive growth model for *Zooglea ramigera* and the filament Eikelboom Type 021N, using experimentally derived kinetic parameters. The model allowed the analysis of the effects of selectors on the growth of filaments and floc formers.

It was observed that pulse feeding has a profound influence on the metabolism of the bacteria (Van den Eynde et al., 1983). Van den Eijnde et al. (1984) has performed experiments with intermittently and continuously fed bacterial cultures for evaluating the effects of feeding pattern on bulking in the activated sludge system. In this study, it was observed that the intermittently fed activated sludge systems had a low sludge volume index (SVI) and high storage capacity and continuously fed systems had a high SVI and low capacity for the accumulation of storage polymers (Van den Eynde et al., 1984). These observations with pure cultures correlated very well with the observation that continuously fed (completely mixed) activated sludge systems had a high SVI and low storage capacity. Despite this recognition of importance of storage

polymers, bulking sludge is usually only explained in terms of the KST. This theory was supported by several studies that point out that the feeding pattern had a strong influence on the kinetics and on the population dynamics of activated sludge. It is believed that microbial populations with a high abundance of filamentous bacteria are predominant in continuously fed completely mixed flow systems compared to continuous fed plug flow type systems (Martins et al., 2003).

Although the Chudoba et al. (1985) stated that at high loading rate the insufficient regeneration of the stored material by the floc forming bacteria leads to the occurrence of a high SVI, Krishna and Van Looshdrect (1999b) have observed an opposite trend as at high substrate loading a high SVI correlates with a low substrate accumulation. There is evidence that floc-formers are competitively advantageous than filamentous bacteria under intermittent feeding or plug-flow conditions because of their ability to store substrates during imposed transients and subsequently reuse them for growth (Beccari et al. 1998; Dircks et al. 2001; Martins et al. 2003; Van den Eynde et al. 1983). This is particularly important because the dynamic nature of substrate flows into wastewater treatment plants elicit transient responses from microorganisms.

2.5.2 Role of storage on the microbial selection

The role of storage under transient condition has been often called as a key factor for understanding the microbial dynamics in activated sludge. Because the storage response is faster than growth response, it has been assumed that a competitive advantage exists, because those microorganisms are most able to store substrates during imposed transients and subsequently reuse them for growth (Majone et al., 1999). Namely, bulking control is usually considered as a matter of competition for available substrates among floc-forming and filamentous microorganisms, based on respective rates of substrate uptake.

Although many external factors such as microbial interactions, time variations in the growth environment, mass transport limitations, etc., may affect the competition, recent studies have suggested that storage phenomena may be more important than kinetics in microbial selection (Majone et al., 1996; Van Loosdrecht et al., 1997). The role of storage on population dynamics has been studied in detail for filamentous bulking control, where selection in favour of floc-formers against filaments is

exerted by selectors or similar process configurations, which introduce a substrate concentration gradient under aerobic or anoxic conditions (Majone et al., 1999).

Filamentous bacteria are supposed to have, besides a lower substrate uptake rate, less capacity to accumulate carbon reserves although they can store substrate under high substrate concentrations. However, there is no agreement whether a lower storage capacity is truly a general feature of filamentous microorganisms and bulking sludge with respect to floc-formers and well settling sludge (Martins et al., 2004). Recent studies showed that bulking sludge could have a high storage capacity as well (Krishna and Van Loosdrecht, 1999a and Martins et al., 2003). A competitive advantage of floc-formers against filaments due to its supposedly higher storage response should therefore not be considered as an absolute rule (Beccari et al., 1998). Beccari et al. (1998) observed that feast and famine conditions created a bulking sludge with a high storage capacity; Martins et al. (2003) also found that there were not different PHB storage rates or yields between bulking and well-settling sludge. On the other hand, Martins et al. (2003) observed that filamentous bacteria are usually no more than 20% of the bacterial population in bulking sludge and therefore small kinetic differences that may exist may be undistinguishable in mixed activated sludge populations.

Beccari et al. (1998) indicated the dominance of the filamentous bacteria (like Type 021N) although the storage response was dominant at the SRT ranging from 5 to 10 sludge ages. They observed the storage of PHB mainly inside of filamentous bacteria with epifluorescence microscopy. Both high storage capacity and resistance to starvation can provide further competitive advantage for these filaments in a large range of operating conditions, thus it is unlikely that kinetic control of such a bulking could be easily achieved by increasing or decreasing organic load or frequency of feed cycles. Independently from being well settling or bulking, the high storage capability of the filamentous organisms *Thiothrix* CT3 (Majone et al., 2007) and *Nostocoida limicola II* belongs to the alpha subclass of Proteobacteria (Dioinisi et al., 2002) has also been reported.

2.5.3 Microbial species responsible for storage in activated sludge

Although, PHA storage capabilities of a lot of pure culture were determined, limited number of study was performed for the detection of the microorganisms responsible

for PHA accumulation in activated sludge systems under aerobic conditions. In the study conducted at the SRT of 1 day with the pulse feeding of mixed substrate (acetate, lactate and propionate), the main genera identified were *Thauera*, *Candidatus Meganema perideroedes* and *Flavobacterium* (Majone et al., 2006). All the mentioned genera have been recovered from activated sludge by various authors (Lee et al. 2003; Valle et al. 2004). The *Cytophaga-Flavobacterium* group has been studied in relation to PHA production under anaerobic/aerobic conditions (Liu et al., 2001), while *Candidatus Meganema perideroedes* has been shown to be present and able to be stored in an aerobic reactor intermittently fed with acetate (Beccari et al., 1998; Levantesi et al., 2004).

In the study conducted by Dionisi et al. (2005) in SBR fed 10 minutes with mixed acid (acetic, lactic and propionic acids) at the SRT of 1 day, the change in the microbial species related to the time of reactor operation have been determined by denaturing-gradient gel electrophoresis (DGGE). The most representative bands of the gel corresponding to day 80 were excised and sequenced. Uncultured *Methylobacteriaceae bacterium clone* M10Ba54 (98% homology), *Flavobacterium sp*. F3 (97% homology), both already described in activated sludge, *Candidatus Meganema perideroedes* strain Gr28 (99% homology), a filamentous organism able to store PHA, and *Thauera chlorobenzoica* (95% homology) were identified. In this study, the species belong to *Thauera spp*. in the beta subclass of proteobacteria was determined as the main responsible for the PHA accumulation. Same researchers have also reported the storage capability of filamentous bacteria *Nostocoida Limicola II* belonging to alphaproteobacteria phylum in the study carried out with anoxic/aerobic cycles at the SRT of 6 days with mixed substrate (acetate, ethanol, glucose and glutamic acid) under 1 minutes of pulse feeding (Dionisi et al., 2002).

The following study of the same researchers (Dionisi et al., 2006) showed that the most abundant taxonomic group was Betaproteobacteria by screening of the clones using amplified ribosomal DNA restriction analysis (ARDRA) after confirming the speciation of the population for PHA accumulation by DGGE. The most abundant taxonomic group obtained was the Betaproteobacteria, in which at least two different species of *Thauera*, two species of *Alcaligenes*, *Comamonas sp*., *Achromobacter sp*., and *Pseudomonas sp*. were present. In this study, other dominant bacteria were reported as *Gammaproteobacteria* group, namely the genera *Kluyvera*,

Pseudomonas, and *Acinetobacter* and *Xanthobacter sp*., belonging to the *Alphaproteobacteria*, and *Curtobacterium sp*. were reported.

A different approach was applied by Serafim et al. (2006). Using fluorescence in situ hybridization (FISH), the dominant organism present in a SBR operated at the SRT for 10 days with the addition of acetate was detected as Azoarcus genus within betaproteobacteria phylum and the PHA storage capability of these bacteria has been confirmed with the Nile blue staining. In the quantitative FISH analysis applied to biomass fed with acetate, the dominant species were identified as *Thauera spp*. and *Azoarcus* in the reactors operated at the SRT of 10 and 1 days (Lemos et. al., 2008). In addition to these species, Amaricoccus species were found quite abundant at the SRT of 10 days, while they were not observed at the SRT of 1 day. The PHA storage capability of all these species was also confirmed with Nile Blue staining. The differences in the dominant PHA accumulating species have shown the effect of the operational conditions on the selection of the microbial populations.

The detected dominant population mainly belongs to *alphaproteobacteria, betaproteobacteria, gammaproteobacteria, cytophaga-flexibacter-bacteroides,* HGC and LGC phyla in the microbial characterization study of the reactor operated with the aim of the phosphorus removal (Liu et al., 2001). Within these species, the bacteria belong to only the beta and gamma subclass of the Proteobacteria which was observed as the responsible from PHA storage by combining FISH with DAPI and Nile Blue Staining procedures.

In the study conducted with the sludge from aerobic-anaerobic SBR by FISH/MAR, the bacteria belonging to the low G+C and high G+C phyla were observed as responsible for anaerobic glucose assimilation, although the bacteria belonging to alpha and gamma subclass of proteobacteria were detected as responsible for PHB production (Kong et al., 2001). In the FISH experiments carried out with the activated sludge samples taken from 11 WWTPs, the cocci in clusters of tetrads short rods in clusters and few amounts of filamentous bacteria were determined as the starch hydrolyzing organisms (SHOs) by starch staining combined with FISH (Xia et al. 2008). Within these bacteria, the filamentous bacteria were determined as the consumers of starch hydrolysates due to the observation of a decrease in the amount of the filamentous bacteria after the addition of the inhibitor for eliminating the consumers of the starch hydrolysates.

3. MATERIAL & METHODS

3.1 Experimental Scheme for the Evaluation of the Dynamic Response in Activated Sludge

The main aim of experimental studies is to evaluate the effect of operating conditions on the storage and growth response of activated sludge by comparing the results gathered from sequencing batch reactors (SBRs) operated at different sludge retention times (SRTs) with different carbon sources. The reason for studying the effects of these conditions can be explained as follows:

- This study was carried out with two different carbon sources: acetate which is the one of the important substrates in domestic wastewater and is stored as PHB and starch which requires hydrolysis by extracellular enzymes prior to its consumption by biomass and is stored as glycogen. Finally, the mixture of these two substrates was used in order to investigate the interaction between fates of two different substrates.
- Two different feeding patterns were applied for the investigation the effect of feeding periods on growth and storage kinetics of activated sludge systems in SBR: pulse and continuous feeding. While dynamic conditions were present in the pulse fed SBR and consequently, storage was expected as main mechanism, biomass would have a time for increasing their specific growth rate and consequently, growth mechanisms could be expected as the main mechanism in continuously fed SBR.
- Since, hardly any information is available on the stoichiometry and kinetics of storage polymer production and consumption related to SRTs and feeding patterns, the growth rate on external substrate needs to be evaluated as a function of the SRT for different feeding conditions. Therefore, in this study, biomass was subjected to two different SRTs in each feeding pattern and carbon source.

In addition to this main experimental plan, additional experiments were carried out for investigating the effect of the organic loading rate (OLR) and inoculum on the storage and growth kinetic of activated sludge systems.

The SBR was chosen as reactor type since it is most suited for the purpose of comparing the different activated sludge configurations. Despite its cyclic operation, it may be operated at steady state for a selected sludge age yielding identical responses in each cycle and reflecting this way the effect of microbial culture history on observed results. Also in each cycle, transient response of all relevant parameters, such as COD, biochemical storage products, oxygen uptake rate, etc. may be individually observed and evaluated. Consequently SBR has been widely used as an experimental tool for different research interests (Orhon et al., 1989).

The activated sludge from domestic wastewater treatment plant was used as inoculum at the beginning of each SBR acclimation study. In order to shorten the acclimation period of SBR, inoculum was initially acclimated to carbon source in question for the selected operation conditions in batch reactor operated in fill and draw mode (Figure 3.1). The PHA and glycogen samples were taken regularly and were preserved for the observation of initial storage polymer content of biomass.

Figure 3.1: Fill and draw reactor systems.

3.1.1 Experimental setup of the SBRs

The experiments were performed in two identical laboratory-scale bioreactors which are operated as SBRs (Figure 3.2). A continuously fed SBR and a pulse fed SBR were operated in parallel, in order to observe the effect of the different feeding patterns on rate and yield of substrate removal as well as on the microbial

composition. Except the feeding pattern, all operating conditions were same in parallel SBRs during the acclimation period and cycle experiments.

Figure 3.2: Bioreactor systems operated as SBRs.

The SBRs were operated six cycles a day with the total cycle length of 240 minutes. The operation of each cycle started with aeration and mixing continuously for 180 minutes as reaction phase; where, after an idle phase of 10 minutes at the beginning of the cycle, the nutrient was added for 5 minutes between the 10^{th} and 15^{th} minute and then substrate was added starting from the 15^{th} minute. In both cases, the fill of substrate was included in the aerobic reaction phase, but the fill was either very quick (1 minute, named "pulse") or slow and lasted for most of the reaction phase (150 minutes out of 180 minutes, named "continuous"). The necessary amount of carbon source was added at a constant flow rate of 1.3 mL/min for 150 minutes or at 200 mL/min for 1 minute, for continuous feeding or pulse feeding, respectively. The excess sludge was withdrawn during the last 2 minutes of the reaction period. The identical phases of typical SBR operation and the duration of these phases were summarized in Table 3.1.

Each SBR had a total reactor volume, V_T, of 2.0 L, where the stationary volume holding settled biomass and the fill volume were 1.0 L each, V_0, and V_F, respectively (V_0/V_F ratio 1.0). In each cycle, the fill volume, V_F, included successive additions of nutrient and carbon sources. The same V_0/V_F ratio was kept in during the operation of the SBR 7 and SBR 8, although the total working volume of the reactor was arranged as 1.2 L (at the end of the fill), with an exchange volume ratio of 0.6 L/cycle. Besides that, since sludge bulking caused settling problems in Set 4, the initial volume was increased to 1.8 L and volume and concentration of both nutrient

and substrate feeding solutions were accordingly adjusted to maintain the chosen organic loading and the chosen substrate: nutrient ratio.

Table 3.1: The phase sequence for parallel SBRs.

Phase	Time in cycle (minute)	
	Pulse	Continuous
Idle (with mixing) phase (T_I)	0-10	0-10
Fill phase (T_{Fn}) (for nutrient)	10-15	10-15
Fill phase (T_{Fs}) (for substrate)	**15-16**	**15-165**
Reaction phase (T_{AX})	15-180	15-180
Excess Sludge withdrawal phase (T_W)	179-180	179-180
Settling phase (T_S)	180-210	180-210
Draw phase (T_D)	210-240	210-240

Aerobic conditions were maintained by mechanical stirring and air bubbling throughout the feeding and reaction phases. Dissolved oxygen was continuously monitored and was never below 2 mg/L during the reaction phase. The temperature of the SBR was kept at 25 ºC and the pH was kept at around 7.0 ± 0.5 with ADI 1010 bio-controller and BioXpert software. The SRT was adjusted according to the daily sludge production, by taking into account the amount of volatile suspended solid (VSS) discharged with both waste sludge withdrawal and suspended solids in the effluent at each cycle.

The substrate feed was prepared by diluting a stock solution of sodium acetate trihydrate ($CH_3COONa.3H_2O$) and/or soluble starch ($(C_6H_{10}O_5)_n$) in distilled water. The nutrient requirements of the activated sludge were supplied with the addition of the Solution A and Solution B (O'Connor, 1972). The composition of these solutions is given in Table 3.2.

Table 3.2. Composition of Solution A and B.

Solution	Component	Amount of Component (g/L)
Solution A	NH_4Cl	120
	KH_2PO_4	160
	K_2HPO_4	320
Solution B	$MgSO_4.7H_2O$	15
	$CaCl_2.7H_2O$	2
	$FeSO_4.7H_2O$	0.5
	$ZnSO_4.7H_2O$	0.5
	$MnSO_4.H_2O$	0.5

For each SBR run, amount of Solution A and B were adjusted to supply 10 mL of solution for 1000 mgCOD/L of carbon source, so to have enough buffer capacity and to supply nitrogen and phosphorus at a non limiting concentration with respect to the carbon source (O'Connor, 1972).

3.1.2 Operating conditions of the SBRs

The SBRs were operated until reaching steady state conditions. In order to verify the establishment of steady-state conditions for each experimental run, the SBR performance was determined by monitoring the dynamic pattern of the dissolved oxygen (DO) during several cycles and by measuring volatile suspended solid (VSS), chemical oxygen demand (COD) and storage polymers (PHA and glycogen) at the end of the cycles periodically. The establishment of SBR steady-state was ascertained through the stability of above reported parameters, after operation for at least 3 SRT, and average values were calculated accordingly for SBR performance.

SBR acclimation studies were performed on 16 of different operating conditions at two different SRTs with three different carbon source compositions inoculated from two different WWTPs in order to investigate the effects of carbon sources, SRTs and sludge histories on growth and storage. As the different SRTs and feeding patterns were applied to SBRs, the biomass concentrations of each system were different under steady state as seen at the steady state operating characteristics of SBRs summarized in Table 3.3.

The fresh inoculum was obtained from the domestic wastewater treatment plants at the start up of each SBR operation excepting the SBR 8. The SBR 1-8 were acclimated to acetate for evaluation of the effect of OLR, SRT and sludge history on substrate removal mechanisms. While the SBR 3 and SBR 4 were operated for the comparison of the different mechanisms at different SRTs under same operating conditions with SBR 1 and SBR 2, the SBR 5 and SBR 6 were operated to compare the effect of OLR on the SBR performances of SBR 3 and SBR 4, respectively. For evaluating the role of the inoculum in the dynamic response and microbial composition of the activated sludge, SBR 7 and SBR 8 were operated in exactly same conditions with SBR 4 and SBR 3, respectively, excepting the inoculum. The inoculum of the SBR 7 and SBR 8 was taken from the Rome Nord domestic wastewater treatment plant, Italy, although the inoculum of all other SBR was

obtained from the ISKI Paşaköy Advanced Biological Wastewater Treatment Plant, Turkey (Figure 3.3).

Table 3.3: The steady state operating characteristics of SBRs.

Set No	Feeding Pattern	Carbon Source	Design SRT (day)	OLR (mgCOD/L.d)	Average VSS (mgVSS/L)
Set 1	Pulse	Acetate	8	1200	1479 ± 12
Set 2	Continuous	Acetate	8	1080	1567 ± 28
Set 3	Pulse	Acetate	2	1440	845 ± 15
Set 4	Continuous	Acetate	2	1500	1408 ± 13
Set 5	Pulse	Acetate	2	2850	1690 ± 11
Set 6	Continuous	Acetate	2	3228	1937 ± 24
Set 7	Continuous	Acetate	2	1560	1380 ± 8
Set 8	Pulse	Acetate	2	1554	1050 ± 14
Set 9	Pulse	Starch	8	1216	2010 ± 14
Set 10	Continuous	Starch	8	1382	1800 ± 11
Set 11	Pulse	Starch	2	1602	1300 ± 13
Set 12	Continuous	Starch	2	1722	1230 ± 10
Set 13	Pulse	Mixed Substrate	8	1200	2380 ± 12
Set 14	Continuous	Mixed Substrate	8	1200	2320 ± 14
Set 15	Pulse	Mixed Substrate	2	1620	1410 ± 11
Set 16	Continuous	Mixed Substrate	2	1620	1300 ± 9

Figure 3.3: Typical appearance of the SBRs at the beginning of the cycle (idle phase).

The first four SBRs operation were replicated with starch (SBRs 9-12) and mixed substrate (SBRs 13-16) under same operating conditions for evaluating the effect of carbon source on dominant microbial processes.

Even though the fresh inoculum was used for each SBR study, the activated sludge of SBR 7 was used as inoculum of SBR 8 so to evaluate the effect of the feeding pattern shift on the storage and growth kinetic of activated sludge. In other words, after steady state was observed in SBR 7 under continuous feeding, SBR performance was characterized with the cycle experiments during typical cycles by feeding in usual continuous mode and by applying a single pulse feeding (three replicates), for providing information on the dynamic response of the biomass acclimated under continuous feeding. Enough time was allowed between replicates of dynamic experiments with pulse feeding, to avoid excessive disturbance of the steady state. Subsequently, after cycle experiments were completed, the SBR feeding pattern was changed from continuous to pulse feeding, all other conditions remaining the same. The same experimental program was adopted as for monitoring the steady state and performing the cycle experiments.

After a steady-state had been established, the dynamic behaviour of the SBRs during the several cycles was monitored by taking several samples for COD, carbon source (acetate and/or glucose) and storage polymer (glycogen and PHA) analyses with 2 minutes intervals in the first 30 minutes and with 10-20 minutes intervals in the rest of the reaction phase. The cycle experiments were carried out as three times in different cycles of the SBRs and average values were calculated accordingly. The measurements of these samples gave information about the substrate removal performances, substrate uptake rates, storage polymer formation/consumption rate and consequently, storage stoichiometry and kinetics of the system for each operating conditions.

3.1.3 Respirometric measurements

Oxygen uptake rate (OUR) profiles were measured within representative cycles at steady-state operation for model evaluation of related substrate removal mechanisms in SBRs. For this purpose, OUR experiments were performed by connecting directly the influent and effluent of a continuous respirometer with measuring frequency of sample per minute (Ra-Combo 1000; Applitek, Nazareth, Belgium) to SBRs. Using the OUR experiments, it was possible to assess individual contribution of different acetate utilization/removal mechanisms to the overall oxygen consumption by means of a single respirometric test. The possible interference of ammonia consumption for nitrification was avoided by inhibiting nitrification with thio-urea. The respirometer

used in the experiments involved a continuous flow-through measurement using the DO concentration in the liquid phase to calculate the respiration rate of the activated sludge (Spanjers et al., 1996).

In principle, the activated sludge was continuously transferred to the respirometer with a peristaltic pump. After passing through the respiration vessel (0.75 L), where the dissolved oxygen at the inlet and outlet were measured by a single DO probe, the sample returned to the SBR system. Hereby the activated sludge was continuously recirculated. Due to the use of a single DO-electrode the measuring frequency was limited by the response rate of the DO-electrode (Spanjers and Klapwijk, 1990).

3.2 Analytical Methods

Although, MLVSS were determined in the aerobic slurry and in the effluent supernatant by using the procedure defined in Standard Methods (1995), the different analytical methods were applied for other measurements in SBRs operated at different laboratories, because of the different facilities of the Istanbul Technical University (ITU) (SBRs 1-6; 9-16) and La Sapienza University (SBRs 7-8).

3.2.1 Analytical methods applied in ITU, Istanbul

Samples for PHA analysis were taken into two 10 mL centrifuge tubes containing 2 drops formaldehyde for preventing the biological activity. The PHA content of the washed (K-P buffer solution) and freeze-dried biomass was subjected to extraction, hydrolization, and esterification in a mixture of hydrochloric acid, 1-propanol, and dichloroethane at 100 ºC (Beun et al., 2000; Çığgın et al., 2009). The resulting organic phase was extracted with water to remove free acids. The extracted propylesters were analyzed by gas chromatograph (Agilent 6890N) equipped with a flame-ionisation detector and capillary column (INNOWAX 19095N-123) where benzoic acid was used as an internal standard for the determination of PHB and PHV contents. The injection split (2:1 ratio) temperature and the FID detector temperature were 50 ˚C and 250 ˚C, respectively. Helium was used as the carrier gas. In the detector, the hydrogen flow was set to 30 mL/min, airflow to 400 mL/min, and the make-up flow (helium) to 25 mL/min. The oven temperature was started at 90 ˚C and kept at this temperature for 0.10 minutes. Then oven temperature was gradually

increased to 150 ˚C and kept at this temperature for 5 minutes and 210 ˚C for 5 minutes.

Acetate samples were also analyzed by gas chromatograph (Agilent 6890N) equipped with a flame-ionisation detector and capillary column of DB-FFAP 125–3232. 1.6 mL of acetate samples filtered through 0.22 μm PVDF syringe filters were transferred into a gas chromatography vial and 0.2 mL of 10M phosphoric acid (H_3PO_4) was added to each vial. For the acetate analysis, the temperature of the injection port and detector were 230 ˚C and 250 ˚C, respectively. The sample is injected with splitless injection. The oven temperature reached 100 ˚C in first 5 minutes and then 160 ˚C; it was kept at this temperature for 5 minutes and fixed at 230 ˚C in 3 minutes. Helium was the carrier gas at 45 mL/min. In addition, hydrogen gas was used at 40 mL/min flow rate.

Samples taken for starch analysis were filtered through 0.45 μm PVDF syringe filters and then were subjected to acidic hydrolysis of starch to glucose by 0.5 mL of 6 M hydrochloride acid (HCl), then the glucose concentration was measured with high pressure liquid chromatograph (HPLC). Glycogen was measured similar to the method described by Smolders et al. (1994), namely, by adding 0.5 mL 6 M HCl to 4.5 mL of homogenized mixed liquor samples, then boiling for 5 h at 100 °C, centrifuging and analyzing the supernatant for glucose content with HPLC. Samples taken for COD were filtered through 0.45 μm PVDF syringe filters and were preserved with H2SO4. The COD samples were measured as described in the ISO6060 method (ISO 6060, 1986).

3.2.2 Analytical methods applied in La Sapienza University, Rome

Samples taken for COD and acetate analyses were filtered through 0.45 μm PVDF syringe filters. The acetate samples were analyzed by gas chromatograph (Perkin-Elmer 8410) equipped with the stationary phase of Carbowax 20 M 4% on CarboPak B-DA. The COD samples were measured with the Spectroquant Kit (Merck).

For PHA measurements, the sludge samples were preserved immediately after sampling with a NaClO solution (7% of active Cl_2). PHA contents of the samples were determined by gas chromatograph after being extracted, hydrolyzed, and esterified to 3-hydroxyacyl methyl esters (Braunegg et al., 1978). The gas chromatograph was equipped with a flame-ionisation detector and 1.83 m of column

was filled with 2% Reoplex 400 on Chromosorb GAW 60-80 mesh with the diameter of 2 mm. Nitrogen was used as the carrier gas. The injection temperature and the FID detector temperature were both 200 ˚C. In the detector, the hydrogen flow was set to 30 mL/min, airflow to 400 mL/min, and the make-up flow (helium) to 25 mL/min. The oven temperature was started at 100 ˚C and kept at this temperature for 1 minute. Then oven temperature gradually increased with the temperature ramp rate of 10 °C/min up to a final Temperature of 150 °C and kept at this temperature for 1 minute.

3.3 Molecular and Metabolic Techniques

3.3.1 FISH analysis

FISH analyses were performed as described by Amann (1990). FISH analysis consists of three phase: *Cell Fixation*, *Hybridization* and *Examination*.

Cell Fixation: The aim of the fixation is the preservation of the morphology and the RNA content of bacteria. An aliquot of sample collected from the each SBR was fixed for later FISH analysis, as summarized below:

- For the *gram negative* bacteria, 0.5 mL of formaldehyde (37%) was added to 4.5 mL of sludge and the sample was kept at 4°C for 1 hour. After 1 hour, 5 mL of cold ethanol (96%) was added to the sample and the sample was stored at -20°C.
- For the *gram positive* bacteria, 5.5 mL of ethanol (96%) was added to 4.5 mL of sludge and the sample was kept at 4°C for 1 hour. After 1 hour the sample was stored at -20°C.

Hybridization: The hybridization procedure can be summarized as below:

- 5 µL of fixed sample was spotted on gelatine-coated side and dried for 10 minutes at 46°C.
- The specimen on the slide was dehydrated in an increasing ethanol series (3 minutes each in 50, 80 and 96% (v/v) ethanol)
- After adding 10 µL of hybridization solution to the each well, the 0.5 µL of probe was added to each well by inserting the pipette tip directly into

hybridization solution. The composition of the hybridization solution is given in Table 3.4.

Table 3.4: Composition of hybridization buffer.

Components	Amount	Comment
5M NaCl	360 µl	It is used for salinity
1M Tris/HCl	40 µl	It is used for buffering
Milli Q		Its amount depends on formamide concentration (Table 3.6)
Formamide		Its amount depends on the strength of the probe (Table 3.6)
10% SDS	2 µl	It should be added at last, after mixing the solution

- Then slide was placed into hybridization chamber and left in hybridization oven at 46 °C for at least 90 min.
- For the next step, the washing buffer was prepared and heated in water bath at 48 °C at least for 30 minutes. The composition of the washing buffer is given in Table 3.5.
- After the hybridization step was completed, both sides of the slide were washed with washing buffer and placed into washing buffer. The washing buffer containing slide was heated in water bath at 48 °C for 10-15 min.
- After the washing step, slide was taken from washing buffer and was gently rinsed with cold double distilled water (milli- Q).

Table 3.5: Composition of washing buffer.

Components	Amount	Comment
5M NaCl		Its amount depends on formamide concentration (Table 3.6)
1M Tris/HCl	1 ml	
Milli Q		Fill until the 50 ml mark
10% SDS	50 µl	It should be added at last, after mixing the solution

- After the slide dried with compressed air or putting to a dark place, an antifading solution including DAPI was dropped to the middle of the wells. The cover glass was placed to the top of the slide and DAPI was disturbed each well equally.

The volume of the formamide, milli Q and sodium chloride used for hybridization and washing buffer is given in Table 3.6 according to the formamide concentrations specified for oligonucleotide probes.

Table 3.6: Volume of NaCl for different formamide concentrations.

% Formamide	Amount of Formamide (µl)	Amount of milli Q (µl) (hybridization solution)	Amount of 5M NaCl (µl) (washing solution)
0	0	1598	9000
5	100	1498	6300
10	200	1398	4500
15	300	1298	3180
20	400	1198	2150
25	500	1098	1490
30	600	998	1020
35	700	898	700
40	800	798	460
45	900	698	300
50	1000	598	180

All the hybridizations with group specific probes were carried out simultaneously with probes EUB338 (Amann et al., 1990), EUB338-II and EUB338-III (Daims et al., 1999) combined in a mixture (EUB338mix) for the detection of most bacteria and with DAPI staining for quantifying the total number of cells. DAPI was added to hybridization buffer at a final concentration of 1 µg/mL. All the probes were synthesized with FITC and Cy3 labels purchased from MWG AG Biotech (Germany). Slides were examined with a microscope (Olympus BX51).

Hybridization studies were performed with the oligonucleotide probes which were previously used for the examination of similar activated sludge systems in order to detect the microbial compositions of the SBRs in a phylum or a group level. The properties of the oligonucleotide probes used for FISH experiments are given in Table 3.7.

Examination: The activated sludge samples were examined with an Olympus BX51 epifluorescence microscope at 1000x magnification and images were captured with Olympus F-View CCD camera and handled with AnalySIS software (SIS, Munster, Germany). The abundance of the diverse groups was expressed as percentage of all

bacteria (as area occupied by probe-binding cells). The standard error of the mean was calculated as the standard deviation divided by the square root of the number of images.

Table 3.7: Properties of the oligonucleotide probes.

Probe Name	Specificity	Formaldehyde Concentration (%)	References
EUBmix (EUB338 I+II+III)	Most Bacteria	20-35	Amann et al., 1990; Daims et al., 1999
ALF968	Alphaproteobacteria	20	Neef, 1997
BET42a	Betaproteobacteria	35	Manz et al., 1992
GAM42a	Gammaproteobacteria	35	Manz et al., 1992
HGC69A	Actinobacteria	25	Roller et al., 1994
LGC354mix	Firmicutes	35	Meier et al., 1999
THAU832	Thauera spp.	35	Loy et al. 2005
AZA645	Most members of the Azoarcus cluster	35	Hess et al. 1997
CF319a	most Flavobacteria, some Bacteroidetes, some Sphingobacteria	35	Manz et al., 1996
CFX1223	phylum Chloroflexi (green nonsulphur bacteria)	35	Björnsson et al., 2002

3.3.2 Staining and quantification procedures

The staining protocol described in Jenkins et al. (2004) was applied for Gram and Neisser staining procedures. The presence of intracellular polymeric storage compounds was evaluated by means of a specific fluorescent Nile Blue dye according to the method developed by Ostle and Holt (1982). An Olympus BX51 microscope was used for the observations of the biomass in phase contrast, bright field (after Gram, Neisser and Nile blue staining) and epifluorescence (after Nile blue staining and FISH).

The application of FISH in combination with staining procedures was aimed to highlight the dominant bacterial groups with storage and/or direct growth capabilities. In some preparations, the chemical staining was performed after FISH analysis as described in Levantesi et al. (2002). The quantification was made with

procedure described by Di Iaconi et al. (2010). The filamentous bacteria were identified according the morphological peculiarities and phylogenetic affiliation estimated by FISH as described in Levantesi et al. (2004).

Post-FISH Nile Blue staining was applied for the identification of the cells containing intracellular polymeric storage compounds (PHA) to SBRs 7 and 8. For this purpose, FISH was carried out and representative images captured, then the cover slip was removed from the slide. The slide was washed with distilled water and dried again by compressed air. Then chemical staining was carried out by Nile Blue (Ostle and Holt, 1982). Fields photographed in FISH were relocated and rephotographed with the chemical stain. For the quantification, the amount of the cell hybridized with specific probe, EUBmix and DAPI were counted on at least twenty different fields of view selected randomly for each probe.

3.3.3 NMR analysis

For evaluating the interaction between the metabolisms of different substrates through Nuclear Magnetic Resonance (NMR), batch experiments were carried out with ^{13}C labelled acetate (Sodium acetate-$^{13}C_2$ 99 atom %, Sigma Aldrich) or ^{13}C labelled starch (starch - ^{13}C from algae 99 atom %, Sigma Aldrich). Any batch experiment with one labelled substrate was repeated in either the presence or absence of the other substrate in the unlabeled form. By this way, the removal mechanism of ^{13}C labelled acetate was evaluated while the unlabeled starch was either present or not. Same procedure was also applied for the ^{13}C labelled starch. In addition, any experiments were performed with unlabelled substrates for taking into account the natural abundance of the ^{13}C substrate.

For the sake of simplicity, batch experiments for NMR analysis were only performed by using the activated sludge that was already acclimated to pulse feeding of mixed substrate (acetate + starch) in the SBR at SRT 8 days namely SBR 13, under steady state conditions.

For each NMR experiment, the appropriate amount of activated sludge was taken from SBR 13 to obtain at least 1000 mg VSS for following NMR analyses. Activated sludge was placed in a respirometer in order to follow the depletion of the substrates in terms of OUR profile whereas no intermediate sampling was performed. After the stable endogenous decay line was observed, the labelled and/or unlabeled carbon

sources were added to the sludge. As soon as the OUR profile indicated that the substrates had been depleted from the external medium, the biomass was immediately centrifuged (11000g, 20 min, 4ºC). After the supernatant was discarded, a mixture of chloroform, methanol, and water (1:2:1) was added to biomass as previously described (Miccheli et al., 1991). The resulting mixture was vortexed for 1 minute and then was placed in an ice bath (2 h, 4°C). The suspension was centrifuged (11000g, 20 minutes, 4ºC) and then three phases were obtained as aqueous, organic and biomass phases. While the aqueous phase was dried under nitrogen stream, the organic and biomass phases were re-extracted overnight (4°C) by adding cold methanol and a 0.9% NaCl/water solution at final proportions of chloroform: methanol and water 2:1:3 (v/v/v). After this re-extraction, organic phase and biomass were dried with nitrogen stream. All three phases were stored at -80ºC until NMR analysis.

The dried biomass samples were examined with solid state Cross-polarization spectrum CP-MAS NMR operated at 13C resonance frequency of 100.62 MHz. Spectra were acquired at a spinning rate of 12 kHz to ensure that all rotational sidebands would fall outside of the spectral region of interest. The spectral parameters were as follows: proton pulse duration, 15 s; contact time, 1.5 ms; recycle spectral width, 29761.91 Hz; number of transients, 19899 and receiver gain was fixed at 362.00.

3.4 Calculations

The following relationships given in Equation 3.1 and Equation 3.2 were used in order to define SBR operating conditions.

$$OLR = \frac{Q_f S_0}{V} \tag{3.1}$$

$$\theta_c = \frac{VX}{Q_w X + Q_{eff} X_e} \tag{3.2}$$

and

$$Q_f = Q_w + Q_{eff} \tag{3.3}$$

The average observed yields, Y_{obs} (gCOD/gCOD), in the SBRs were calculated according to the following relationship:

$$Y_{obs} = \frac{Q_w X + Q_{eff} X_e}{Q(S_0 - S)} = \frac{HRT \cdot X}{\theta_c (S_0 - S)} \quad \textbf{(3.4)}$$

Specific substrate removal mechanism associated for each SBR operation was assessed by observing the concentration profiles of COD, storage products (glycogen and PHB) and dissolved oxygen within the selected cycles. The specific acetate removal rates ($-q_S$, mgCOD/mgCOD.h) were calculated in different ways, depending on pulse or continuous feeding. Under pulse feeding, the specific rate was calculated by linear regression of experimental acetate profile and by dividing the obtained slope for the initial biomass concentration. On the other hand, negligible starch profile in terms of glucose was detected due to either the rapidly adsorption of starch onto biomass (Karahan et al., 2005) or the substrate limitation in continuously fed SBRs. Therefore, the specific starch removal rates ($-q_S$, mgCOD/mgCOD.h) were obtained by dividing the organic loading per cycle for the duration of the feast phase and for the initial biomass concentration in SBRs fed with starch.

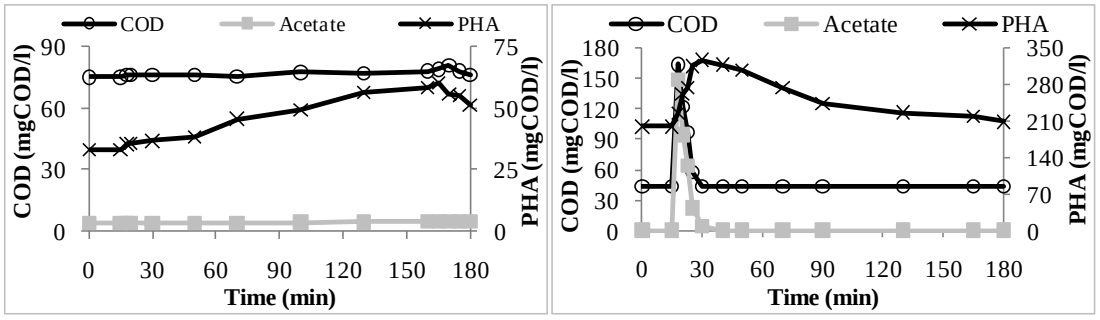
In all SBRs, the specific storage product formation and consumption rates ($q_{p(g)}$ and $-q_{p(g)}$, mgCOD/mgCOD.h) were calculated by linear regression of experimental storage polymer profiles and by dividing the obtained slope for the initial biomass concentration. The substrate fraction used for storage was calculated by dividing the specific storage polymer production rate to the specific substrate removal rate ($q_{p(g)}/-q_S$, adimensional). Under continuous feeding, the experimental profiles were also corrected by taking into account the progressive increase of volume during the reaction phase. Based on literature (Stanier et al., 1976), the simplifying assumption was preliminarily made that PHA was only coming from acetate as well as glycogen was only coming from starch.

4. EFFECT OF DYNAMIC ACETATE FEEDING ON THE SUBSTRATE REMOVAL MECHANISM AND MICROBIAL COMPOSITION OF ACTIVATED SLUDGE

4.1 The Role of the Feeding Pattern, OLR and SRT on the Performance and Microbial Composition of SBRs

4.1.1 Effect of feeding pattern on the performance of SBRs

The dissolved COD, acetate and PHA profiles during a typical SBR cycle are given in Figure 4.1 for SBRs operated at SRT of 8 days (SBR 1 and SBR 2) and in Figure 4.2 for SBRs operated at SRT of 2 days (SBRs 3-6) with acetate.



(a) (b)

Figure 4.1: Acetate, dissolved COD and PHA (secondary axis) profiles of (a) SBR 1 and (b) SBR 2.

At each SRT or OLR, a different behaviour was observed because of the different feeding patterns (pulse vs. continuous feed). As seen from Figure 4.1a, Figure 4.2a and Figure 4.2c, acetate concentration increased very quickly under pulse feeding; then decreased back until its depletion, at times ranging from 15 to 90 minutes from the feed start. Thus, there was no acetate for the remaining part of the cycle and typical "feast and famine" conditions were created. On the other hand, no acetate peak was observed under continuous feeding (Figure 4.1b, Figure 4.2b and Figure 4.2d) because acetate was slowly supplied for most of the cycle and its concentration was always under detection level. Thus, the "feast" phase under continuous feeding was quite longer and less strong than under pulse feeding.

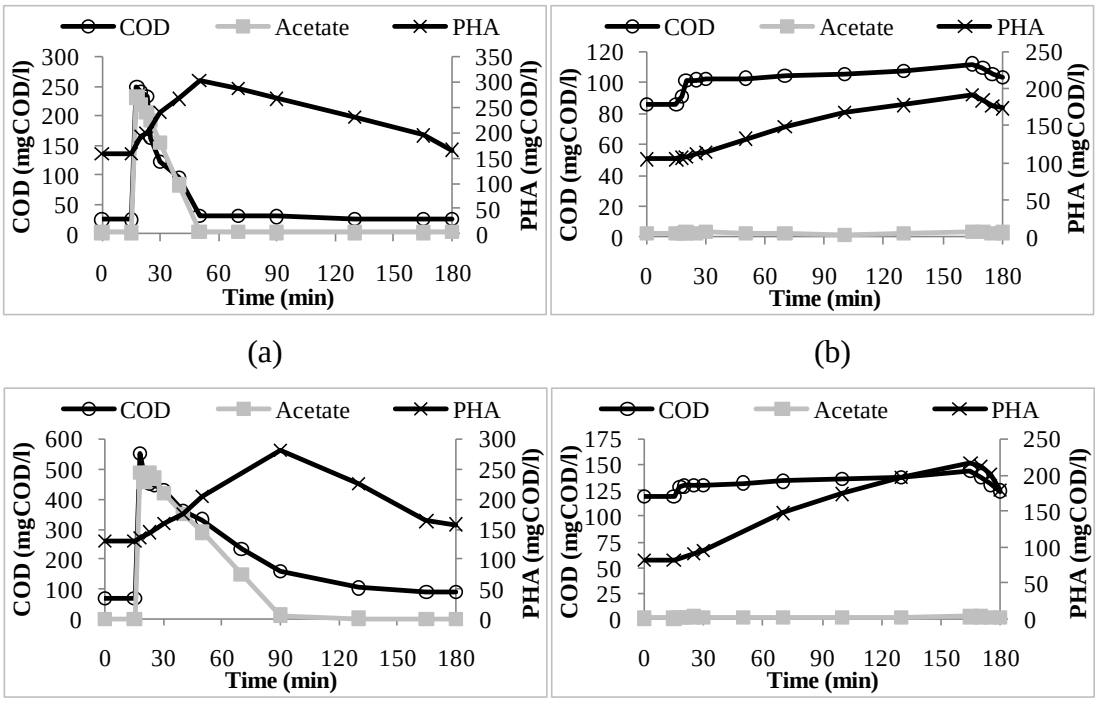


Figure 4.2: Acetate, dissolved COD and PHA (secondary axis) profiles of (a) SBR 3, (b) SBR 4, (c) SBR 5 and (d) SBR 6.

During the feast phase under pulse feeding, the acetate uptake rate was at its maximum because of the excess substrate (see the linear slope of acetate concentration) whereas acetate uptake rate was externally controlled by the substrate feeding at a quite lower rate under continuous feeding. Table 4.1 shows the average rates and yields that were obtained from at least 3 replicate experiments in different cycles, during steady state performance of the SBR. The maximum acetate uptake rate was in the range 158-381 mgCOD/gCOD.h under pulse feeding whereas it was in the range 32-78 mgCOD/gCOD.h under continuous feeding.

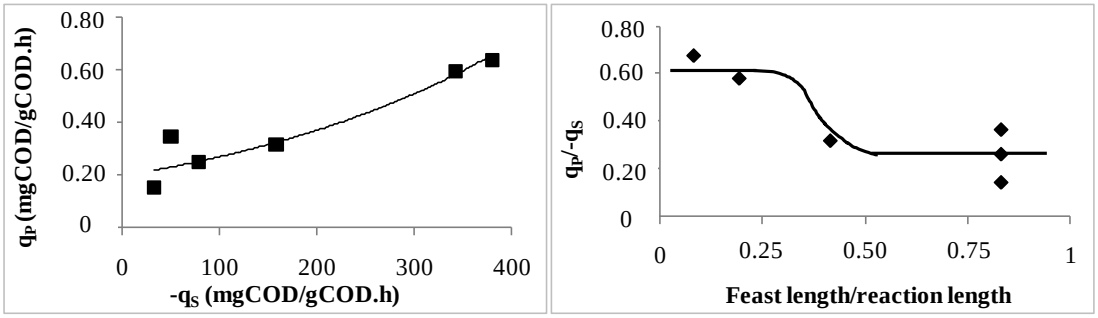
Table 4.1: Average rates and yields observed in cycle experiments.

Parameter	SBR 1	SBR 2	SBR 3	SBR 4	SBR 5	SBR 6
Specific Substrate Uptake Rate, $-q_s$ (mgCOD/gCOD.h)	381	32	343	50	158	78
Specific PHA Production Rate, q_p (mgCOD/gCOD.h)	257	5	199	18	51	21
Specific PHA Consumption Rate, $-q_p$ (mgCOD/gCOD.h)	23	15	51	37	36	55
$q_p/-q_s$	0.68	0.15	0.58	0.37	0.32	0.26

As expected, the stronger "feast and famine" conditions and the higher acetate uptake rates caused a higher PHA production under pulse feeding than under continuous feeding (all other conditions being the same). The PHA specific production rate was in the range 51-257 mgCOD/gCOD.h and 5-21 mgCOD/gCOD.h, under pulse and continuous feeding, respectively. The fraction of the substrate stored into PHA (i.e. the $q_p/-q_s$ ratio in Table 4.1) was higher under pulse feeding than in the parallel run under continuous feeding (all other experimental conditions being the same).

It is noteworthy that PHA storage was anyway present also under continuous feeding; the $q_p/-q_s$ ratio under continuous feeding was never negligible (range 0.15-0.36) and in one condition, it almost matched the one under pulse condition (SRT of 2 days at the highest loading). These evidences suggest that even the small famine period at the end of the reaction phase under aerobic conditions (15 minutes out of 180 minutes, plus settling and idle) was enough to create an unbalanced growth condition and trigger for some storage. On the contrary, no significant PHA storage was reported in the case of the acclimation of bacteria to continuous feeding under anoxic conditions (Çığgın et al., 2007).

Figure 4.3 confirms that feast and famine conditions were generally stronger under pulse conditions and caused that storage was more important during acetate uptake. The PHA storage rate increased as the acetate uptake rate increased (Figure 4.3a) and correspondingly the length of feast phase became a lower fraction of the overall reaction length. Consequently, the $q_p/-q_s$ ratio increased as the feast length decreased (Figure 4.3b).



(a) (b)

Figure 4.3: a) Specific PHA production rate (q_P) as a function of specific substrate consumption rate ($-q_S$) and b) $q_P/-q_S$ ratio as a function of feast phase/ overall reaction ratio.

Accordingly, Figure 4.3b shows that the storage fraction decreases as the ratio between the feast length and cycle length decreases. This is coherent with previous studies reporting that, in order to have a strong storage response, the feast period should be no more than 25-30% of overall reaction period (Dionisi et al., 2001). Interestingly, the pulse feeding at both low SRT and high organic loading (SBR 5) was not able to create a strong feast and famine conditions: indeed the feast/overall reaction ratio was 0.42 and so the importance of storage was more similar to continuous feeding. The average performances of each SBR run are summarized in Table 4.2 (data are the average of measured values from several cycles, under steady state performance of the SBRs).

Table 4.2: SBR Runs: a) Operating conditions and b) Steady state behaviour.

	SBR 1	**SBR 2**	**SBR 3**	**SBR 4**	**SBR 5**	**SBR 6**
a) Operating conditions						
Feeding Pattern	Pulse	Continuous	Pulse	Continuous	Pulse	Continuous
Experimental SRT (d)	7.78	8.10	2.09	2.13	1.91	1.96
OLR (mgCOD/L.d)	1200	1080	1440	1500	2850	3228
b) Steady state parameters						
VSS (mgVSS/L)	1479	1567	845	1408	1690	1937
F/M (mgCOD/mgCOD.d)	0.57	0.49	1.20	0.75	1.19	1.17
Y_{OBS} (gCOD/gCOD)	0.22	0.25	0.40	0.62	0.44	0.44
Feast Phase (m)	15	150	35	150	75	150
PHA Pool (mgCOD/L)	199	33	159	105	130	82
Amount of Stored PHA (mgCOD/L.cycle)	128	27	143	87	150	135
COD fraction used for storage (%)	0.64	0.15	0.60	0.35	0.32	0.25
COD Removal (%)	89	79	95	79	91	88

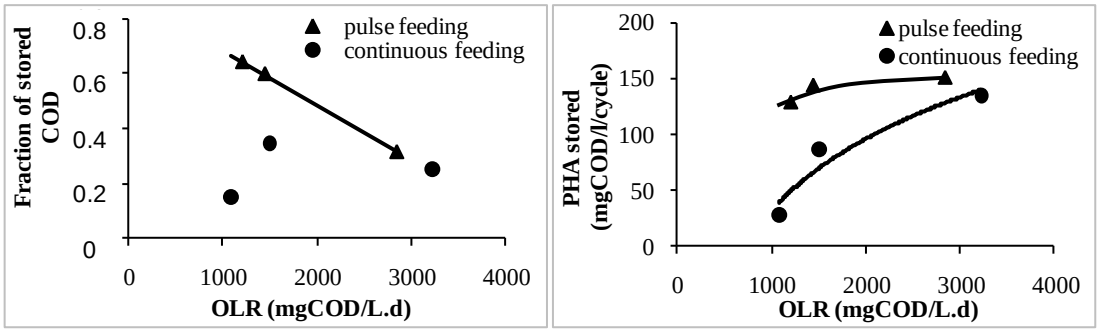
Table 4.2 confirms that pulse feeding caused more PHA storage than continuous feeding (all other condition being the same). As an example, the PHA pool (i.e. the amount of PHA remaining in the reactor at the end of reaction phase) was in the range of 130-199 mgCOD/L and 33-105 mgCOD/L under pulse and continuous feeding, respectively. The fraction of COD used for the storage under pulse vs. continuous feeding was quite different in runs at low organic loading (range 0.60-

0.64 in SBRs 1-3 vs. 0.15-0.37 in SBRs 2-4, respectively) whereas difference was less evident at higher organic loading (0.32 in SBR 5 vs. 0.25 in SBR 6).

Higher observed yields were obtained under continuous feeding than under pulse feeding. This is coherent with the finding that the unbalanced growth through intermediate storage is thermodynamically less efficient than balanced growth with no storage (Van Aalst-van Leeuwen et al., 1997). On the other hand, the percentage of COD removal was higher under pulse feeding than under continuous feeding. Because the acetate was the only carbon source and it was fully removed, less COD removal indicates a higher COD release from cell decay, under continuous feeding than under pulse feeding.

4.1.2 Effect of SRT and OLR on the performance of SBRs

Under pulse feeding, the fraction of COD used for PHA storage decreased very little as the SRT decreased from 8 days to 2 days whereas it strongly decreased as the organic loading increased (Figure 4.4a). As a result, the amount of PHA produced per cycle under pulse feeding was almost unaffected by the OLR (Figure 4.4b). The decrease of $q_p/-q_s$ ratio with the decrease of SRT (at the same OLR) was reported by Beun et al. (2000) and Martins et al. (2003). Dionisi et al. (2001) also reported that $q_p/-q_s$ ratio decreased as the OLR increased (at the same SRT).



(a) (b)

Figure 4.4: Influence of organic loading rate on a) the fraction of stored COD and b) the amount of PHA formed per cycle.

An opposite trend was shown under continuous feeding, because both the fraction of stored COD and the amount of PHA produced per cycle increased as the OLR increased (Figures 4.4a and 4.4b). As a result, the difference between storage responses under pulse and continuous feeding decreased as the OLR increased. This opposite behaviour can likely be explained in the following ways:

a) under pulse feeding, the length of feast/famine ratio is not “a priori” established because it is based on applied organic loading and maximum specific rate of substrate removal; thus the length of feast/famine ratio increases as the organic loading increases and consequently the storage role decreases.

b) under continuous feeding, the feast/famine ratio is directly fixed through the length of the feeding phase; thus the increasing OLR has no effect on the length of the feast/famine ratio whereas it increases the strength of kinetic pressure (because it increases the rate of substrate removal during the fixed feast phase), causing more storage.

However, it remains that the storage was always more important under pulse feeding than under continuous, when other conditions were the same.

4.1.3 Molecular characterization by FISH analysis

4.1.3.1 General description of microbial composition

The main microbial components of mixed biomass sampled from each run were estimated by Fluorescence in Situ Hybridization (FISH). Most of Bacteria were identified by probes specific for the main phyla within Bacteria domain and their distribution in each run is given in Figure 4.5.

As shown in Figure 4.5, *Betaproteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, and members of the *Cytophaga–Flavobacteria–Bacteroidetes* group were retrieved under several operating conditions, while a negligible presence of *Firmicutes* and *Chloroflexi* was observed. Significant differences in microbial composition are evident in sets operated at different SRTs. While *Betaproteobacteria* were more abundant in SBRs operating at SRT of 8 days (samples from SBR 1 and SBR 2), *Alphaproteobacteria* became dominant at lower SRT (SRT 2 days, samples from SBR 3, SBR 4, SBR 5 and SBR 6). Most of *Betaproteobacteria* in SBRs operated at SRT of 8 days were identified as members of *Thauera/Azoarcus* group (probes AZA645 and THAU832).

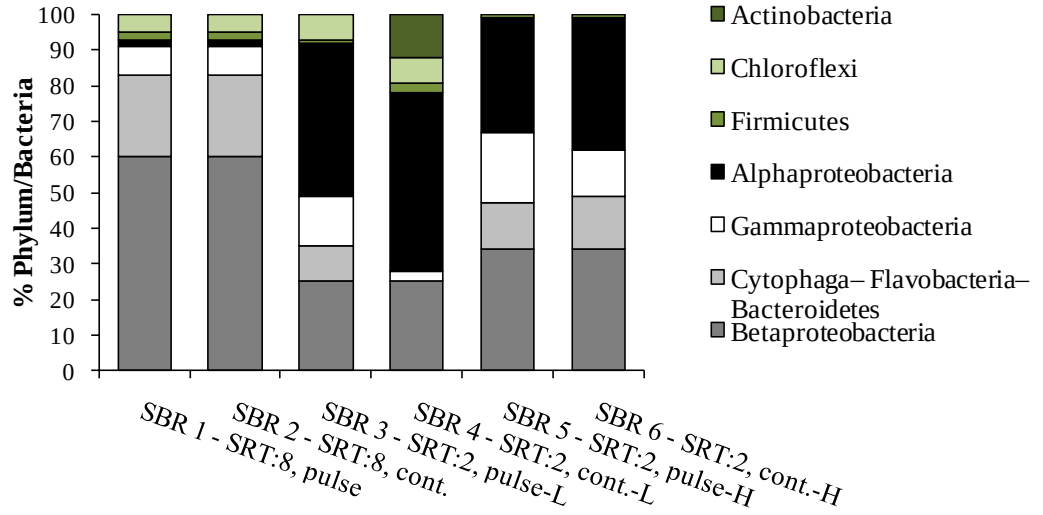


Figure 4.5: Microbial population dynamics estimated by FISH as a percentage of total bacteria in SBRs (L: low OLR; H: high OLR).

Distinctively, the presence of *Actinobacteria* was observed only in SBR 4. A portion of *Alphaproteobacteria* in SBR 5 and SBR 6 samples (about 65% and 55% of *Alphaproteobacteria*, respectively) is characterized by very low fluorescence signals, most likely associated with poorly active or inactive cells. As detailed in the following paragraph, this group was mainly constituted by filamentous bacteria clearly showing empty or deformed cells within the thricoma. The phylogenetic affiliation of filamentous bacteria detectable by FISH is shown in Figure 4.6.

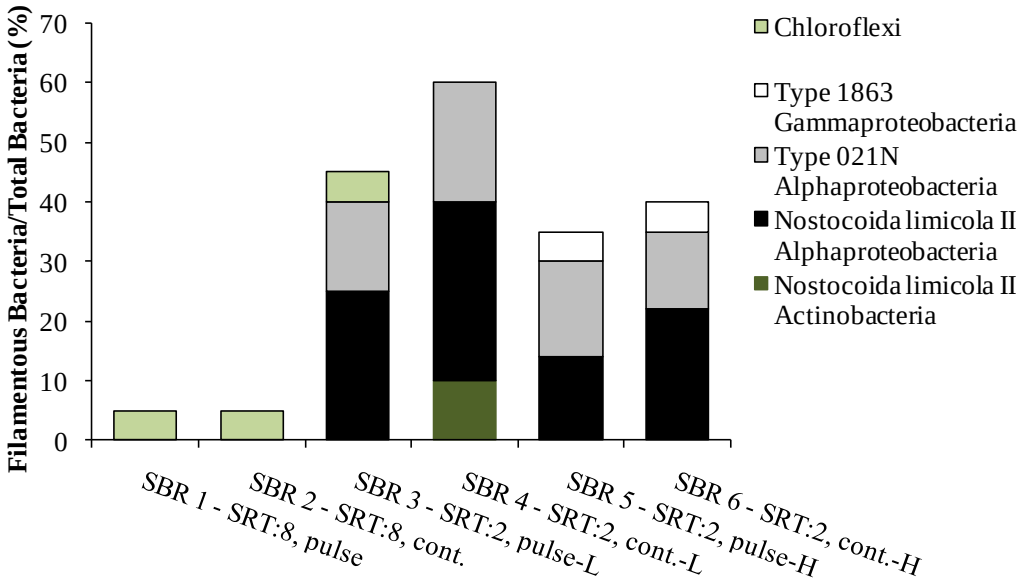
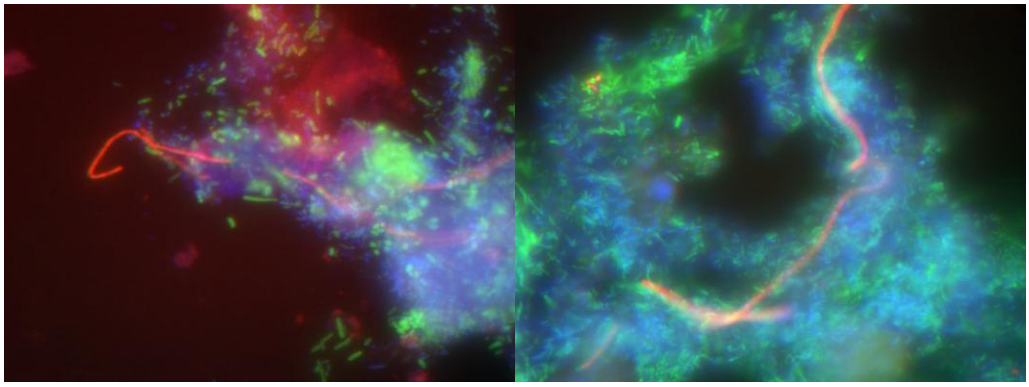


Figure 4.6: Amount of filamentous bacteria estimated by FISH analysis as a percentage of total bacteria in SBRs (L: low OLR; H: high OLR).

The representative pictures of the filamentous bacteria detected in the SBRs operated at the SRT of 8 days are shown in Figure 4.7. The microscopic analysis showed marked differences in the ratio of floc-formers/filamentous bacteria: SBR 1 and SBR 2 operated at SRT of 8 days were mainly composed by microbial aggregates of floc-forming bacteria while all the SBRs operated at SRT of 2 days (SBRs from 3 to 6) were colonized by filaments. The higher amount of filamentous bacteria is in good accordance with bulking problems usually observed in all SBRs operated at the SRT of 2 days, whereas no bulking problems were experienced at SRT of 8 days where filaments were less abundant. Most severe bulking problems were encountered in SBR 4, where the filamentous bacteria were observed in all flocs and their abundance was scored as "dominant" according to Jenkins et al. (2004).



(a) (b)

Figure 4.7: FISH Results for (a) SBR 1 and (b) SBR 2; DAPI (blue) EUBmix (green) and (a) Chloroflexi sp. (red).

Type 021N and *Nostocoida limicola* (*Alphaproteobacteria*) were observed in SBRs from 3 to 6. However, in SBRs 5 and 6, most of these filaments were only visible when the samples were analyzed by phase contrast microscopy and were hardly detectable by FISH, due to the low fluorescence signals of the hybridized cells. In SBR 5 and SBR 6, the presence of the Type 1863 (*Acinetobacter spp.*) belonging to *Gammaproteobacteria* was also detected. Strains of this filamentous bacterium are characterized by maximum growth rate (Tomei et al., 1999, μ_{max}= 9.6 1/d) higher than for *alphaproteobacterial* filaments retrieved in SBR 3 and SBR 4 (Levantesi et al., 2004). The occurrence of Type 1863 (*Acinetobacter spp.*) is therefore in agreement with both the higher organic loading and the dominance of the growth in SBRs 5 and 6. FISH images of the main filamentous bacteria observed at different SBRs are shown in Figure 4.8. The strongly bulking sludge in SBR 4 also contained

filaments of *Nostocoida limicola* belonging to *Actinobacteria*, not detected in any samples from other sets.

4.1.3.2 Effect of feeding pattern on microbial composition

In spite of the strong effect on the storage during the substrate removal, the feeding pattern (pulse vs. continuous feeding) did not appear to have a strong impact on the microbial composition (all other conditions being the same). Based on Figures 4.5 and Figure 4.6, it appears that pulse vs. continuous feeding neither significantly affect the microbial composition in terms of main phyla within Bacteria domain nor of most abundant filaments (with the exception of *Actinobacteria* in SBR 4). This preliminary evidence was also confirmed by looking at which microorganisms were responsible for storage during each set, based on Nile Blue staining (Table 4.3 and Figure 4.9).

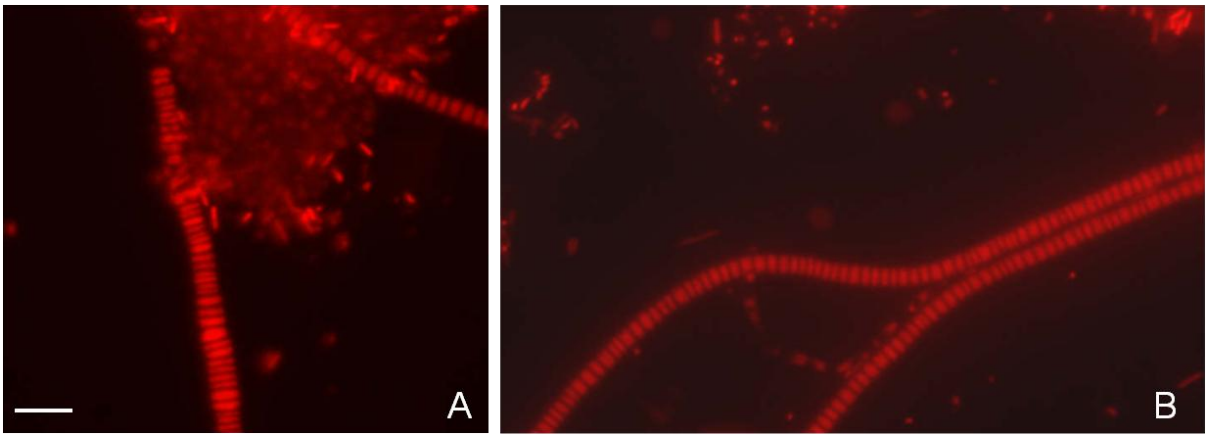


Figure 4.8: Main filamentous bacteria (A) *alphaproteobacterial* Type 021N (B) *alphaproteobacterial N. limicola*. Bar: 5 μm.

At the SRT of 8 days (SBRs 1 and 2), *Thauera/Azoarcus spp.* was observed as the most abundant group among dominant *Betaproteobacteria* and simultaneously main responsible of storage, as highlighted by Nile Blue staining (Figure 4.9).

The participation of *Thauera/Azoarcus* group in PHA storage under pulse feeding has also been reported (Dionisi et al., 2005; Serafim et al., 2006), but the possible change of microbial composition under continuous feeding was not investigated. Our results clearly show that the dominance of *Thauera/Azoarcus* group was independent from the strong difference in stored amount of PHA between pulse and continuously feeding conditions. In other words, *Thauera/Azoarcus* group was able to remove the substrate with more or less storage, depending on the feeding pattern, but this pattern

did not have influence on predominance of *Thauera/Azoarcus* group at the given operating condition (OLR and SRT).

Table 4.3: Main bacteria involved in PHA storage in each SBR.

SRT	Feeding Conditions	OLR	PHA Accumulation Mostly on	Main Bacteria Responsible for Storage
8	Pulse	low	Floc formers	*Thauera/Azoarcus* group
8	Continuous	low	Floc formers	*Thauera/Azoarcus* group
2	Pulse	low	Filamentous bacteria	Type 021N, *N. limicola* (*alphaproteobacteria*)
2	Continuous	low	Filamentous bacteria	Type 021N, *N. limicola* (*alphaproteobacteria*)
2	Pulse	high	Filamentous bacteria	Type 021N, *N. limicola* (*alphaproteobacteria*)
2	Continuous	high	Filamentous bacteria	Type 021N, *N. limicola* (*alphaproteobacteria*)

At the SRT of 2 days, the PHA storage was mainly detected in filamentous bacteria *Nostocoida limicola* and Type 021N, belonging to *Alphaproteobacteria*. Both filaments have been found dominant in industrial WWTPs and shown to be able to aerobically store PHA (Levantesi et al., 2004). Under pulse feeding conditions, the dominance of Type 021N filaments at the SRT of 3 days was reported under aerobic conditions (Beccari et al., 1998) as well as the occurrence of *alphaproteobacterial Nostocoida limicola* at the SRT of 6 days under anoxic/aerobic conditions (Dionisi et al., 2002). In this research, Nile blue staining confirmed that the dominant filaments showed a higher PHA accumulation under pulse operating condition; thus same filamentous bacteria were dominant independently from the extent of the storage and the feeding pattern (other conditions being the same).

Moreover, under similar OLR, pulse feeding was selected for well settling sludge at SRT of 8 days (SBR 1), whereas it was selected for a bulking sludge at SRT of 2 days (SBR 3). In both cases the storage was the main mechanism of quick substrate removal by dominating species, i.e. by floc formers or by filaments, respectively. In accordance with these results, Beccari et al. (1998) observed that feast and famine conditions created a bulking sludge with a high storage capacity. Martins et al. (2003) also found nearly same PHA storage rates or yields in bulking and well-settling sludge.

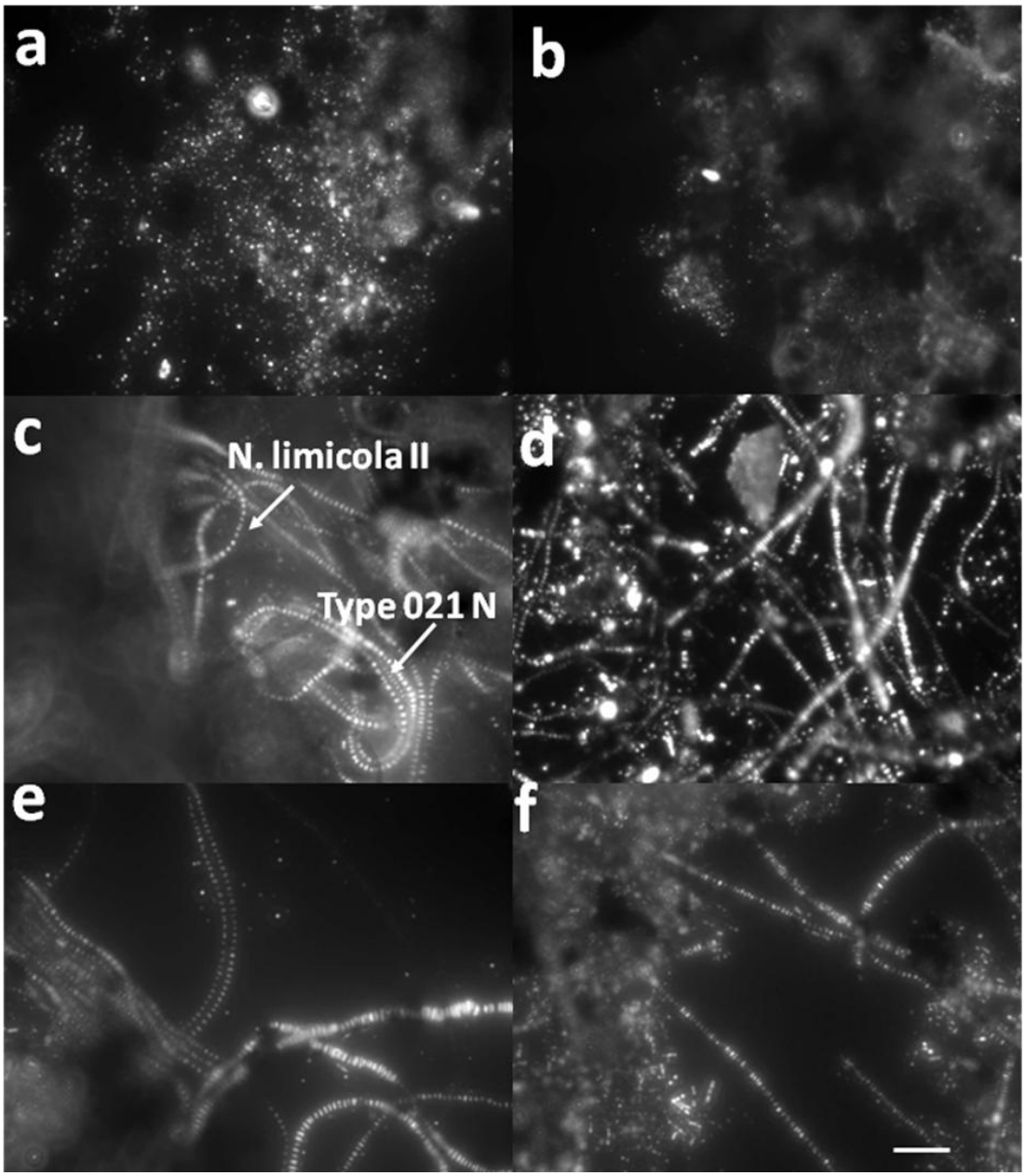
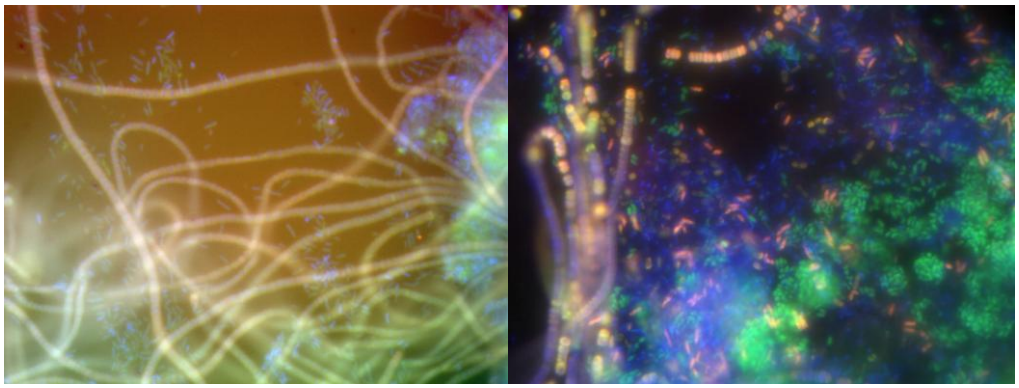


Figure 4.9: Intracellular PHA inclusions in SBRs samples after Nile Blue staining (a) SBR 1, (b) SBR 2, (c) SBR 3, (d) SBR 4, (e) SBR 5 and (f) SBR 6. Bar is 5 μm.

The retrieved similarity of microbial composition in sets operated at same SRT and OLR but under different feeding patterns suggests that the latter has a minor role on microbial composition. Same bacteria were selected at the same SRT and OLR, even though they played more or less role in storage and/or growth in accordance with the applied feeding conditions. This was probably due to the physiological adaptation of dominant microorganisms, as already reported by pure culture studies with several microorganisms (Aulenta et al. 2003; Majone et al., 2007; Insel et al., 2007): these

studies showed that different growth conditions caused a different role of storage and growth during substrate removal, even in the presence of a single species.

Finally, at SRT of 2 days, filamentous bacteria (*alphaproteobacterial* Type 021N and *Nostocoida limicola*), were characterized by weak FISH fluorescence signals due to the low ribosome content, when the system was operated at higher loading as shown in Figure 4.10. The lower storage response at high loading may be most likely explained with the inactivity of these filamentous bacteria, which were the main responsible of PHA accumulation under lower organic loading.



(a) (b)

Figure 4.10: The filamentous bacteria characterized by weak FISH fluorescence signals in (a) SBR 5 and (b) SBR 6.

4.2 The Role of the Inoculum on the Performance and Microbial Composition of SBRs

4.2.1 Comparison of the steady state performances under different feeding conditions

Starting from the inoculation of the SBR 7 with the fresh inoculum obtained from Roma Nord Domestic Wastewater Treatment Plant, the performance of the activated sludge during SBR operation was monitored in terms of the VSS and PHA concentration as a function of SBR operation time as given in Figure 4.11. Left part of the figure refers to continuous feeding (SBR 7) whereas second part refers to pulse feeding (SBR 8). The shift from continuous to pulse feeding was performed at the 35^{th} day of operation, while all other conditions were maintained same.

After nearly 10 days of operation from SBR start up, a steady state was reached under continuous feeding in SBR 7; average concentration of VSS and PHA was 1380 mgVSS/L and 108 mgCOD/L, respectively. The shift from continuous to pulse feeding caused a new transient period of about 10 days where VSS concentration

decreased, whereas PHA concentration increased. After a new steady state was reached at day 45 in SBR 8, average concentrations of VSS and PHA were determined as 1050 mgVSS/L and 170 mgCOD/L, respectively (i.e. a 24% decrease and a 63% increase, respectively). During operation under continuous feeding, sludge settling worsened and the sludge volume index (SVI) strongly increased to nearly 500 mg/L, although the SVI of the inoculum was lower than 120 mg/L. After the shift from continuous to pulse feeding, the sludge settling property quickly improved (SVI<120 mg/L).

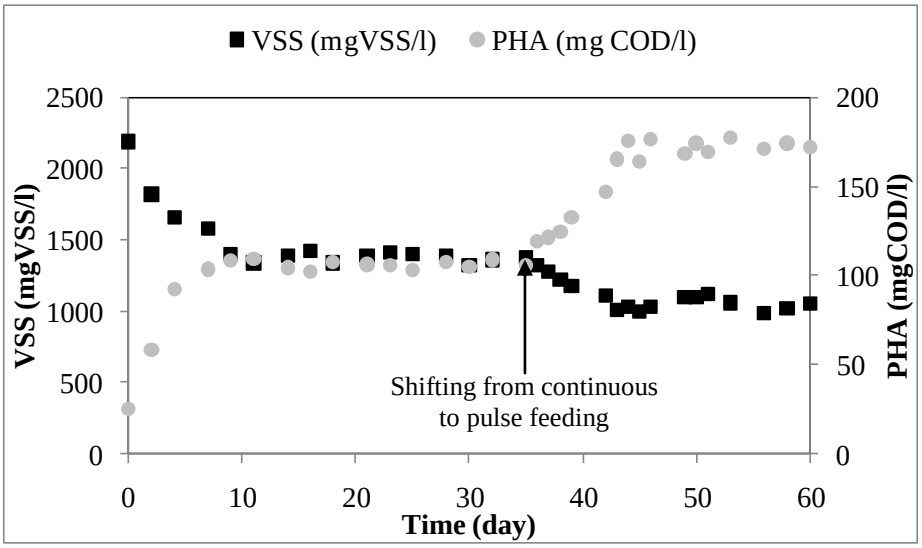


Figure 4.11: Time profiles of VSS (primary axis) and PHA (secondary axis) concentration during the acclimation period.

The average data obtained for different feeding patterns under steady state conditions are summarized in Table 4.4. Based on the higher VSS concentration, the observed yield was also higher under continuous feeding than under pulse feeding. Indeed, as previously reported, the presence of the storage is thermodynamically unfavourable compared to balanced growth response (Van Aalst-van Leeuwen et al., 1997). As expected, based on higher PHA concentration, the amount of the carbon source used for PHA storage was also higher under pulse feeding than under continuous feeding. In addition, the amount of residual soluble COD under pulse feeding was less than under continuous feeding.

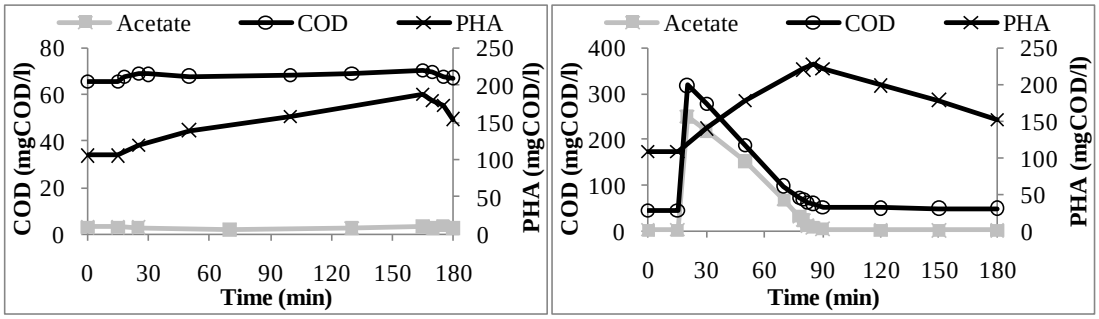
The observed differences, as obtained by shifting from continuous to pulse feeding in the same SBR, are in agreement with previous results obtained from SBRs 1-4, where the comparison between continuous and pulse feeding was performed in parallel by simultaneously starting up two identical SBRs with fresh inoculum.

Table 4.4: Steady State Performances under different acclimation conditions.

Parameter	SBR 7	SBR 8
Acclimation Condition	Continuous	Pulse
SRT (d)	2	2
OLR (mgCOD/L.d)	1560	1554
Average VSS (mgVSS/L)	1380 ± 10	1050 ± 14
Y_{OBS} (gCOD/gCOD)	0.55	0.36
Feast Phase (min)	150	45
Amount of Stored PHA (mgCOD/L.cycle)	83	141
COD Removal (%)	78	90

4.2.2 Comparison of the dynamic behaviour of activated sludge under different feeding conditions

After the establishment of steady state conditions under continuous feeding, cycle experiments were conducted by monitoring relevant parameters during a typical SBR cycle. Experiments were performed by applying either usual continuous feeding which SBR biomass was already acclimated to or a single disturbance of usual conditions by using pulse feeding. The average profiles (three replicates) observed during both types of kinetic experiments is given in Figure 4.12.



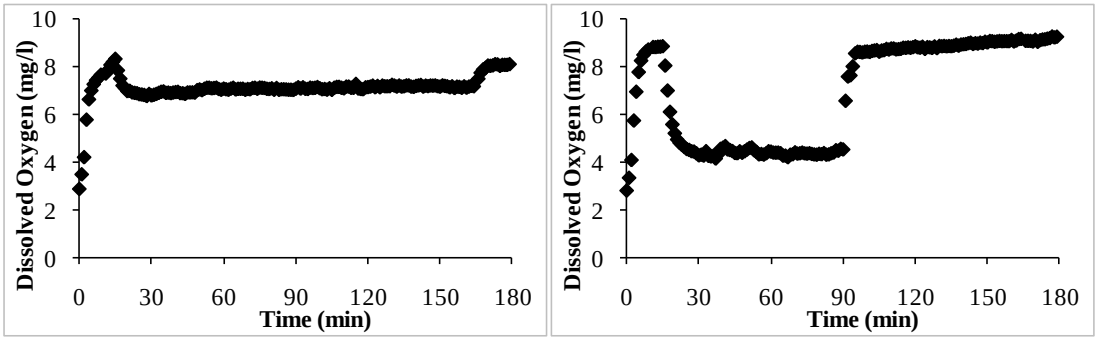
(a) (b)

Figure 4.12: Dynamic behaviour of biomass acclimated to continuous feeding in SBR 7 when applying (a) usual continuous feeding and (b) single disturbance by pulse feeding.

As expected, during experiments with the usual continuous feeding, COD remained almost constant and acetate was always very low. Thus, the acetate removal rate was fully controlled from the acetate loading rate, i.e. from feed flow rate. In spite of the fact that acetate was removed at low concentration and rate, a gradual increase of PHA concentration was observed during the long feeding period as well as a corresponding decrease during the short no-feed period. The PHA concentration

shifted from 106 mgCOD/L to 189 mgCOD/L, corresponding to about one third of acetate load being converted to PHA.

On the other hand, when pulse feeding was applied for just one cycle, the biomass acclimated to continuous feeding conditions; typical "feast and famine" conditions were created. A peak substrate concentration was observed in a few minutes which were totally removed in about 90 minutes. The relative ratio of feast and famine period was 11 under continuous feeding as dictated by substrate loading, although this ratio was 1 when pulse feeding was applied for just one cycle, which was also validated with the dissolved oxygen profiles given in Figure 4.13.



(a) (b)

Figure 4.13: Dissolved oxygen profile of biomass acclimated to continuous feeding SBR 7 when applying (a) usual continuous feeding and (b) single disturbance by pulse feeding.

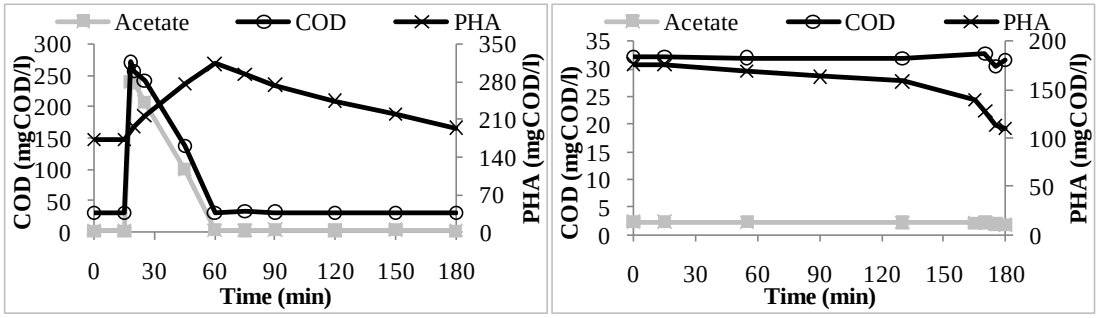
Accordingly, more PHA storage was observed when single pulse feeding was applied instead of the usual continuous feeding (119 mgCOD/L after 90 minutes versus 83 mgCOD/L after 150 minutes, respectively). It is interesting to observe that PHA consumption was also active for a longer period; thus, residual PHA at the end of reaction phase was quite similar under both conditions.

The ability of microorganisms to quickly increase their acetate removal rate and shift it towards more storage under aerobic conditions has been largely reported for pure and mixed cultures (Majone and Tandoi, 2002) as a function of their previous history, e.g. hydraulic residence time in the chemostat.

Similar kinetic experiments where performed after the SBR biomass was acclimated to pulse feeding and a new steady state was reached. As above reported (Figure 4.11), the new steady state in SBR 8 was characterized from a lower VSS and a higher PHA concentration compared to the previous acclimation. The average results

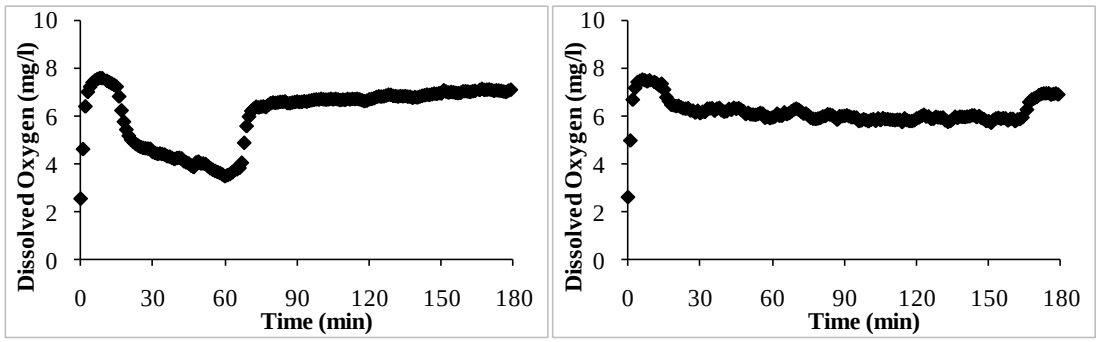
(three replicates) of kinetic experiments obtained by carrying out either pulse feeding or continuous feeding with the biomass acclimated to pulse feeding are given in Figure 4.14 and Figure 4.15. In this case, pulse feeding was the usual procedure, whereas continuous feeding was applied as a single disturbance of previous steady state.

Cycle experiments performed under pulse feeding conditions resulted in similar "feast and famine" profiles with biomass acclimated to either continuous or pulse feeding (Fig. 4.12b and Figure 4.14a, respectively); however, the amount of carbon source used for storage further increased from 46% to 54% when the biomass had been already acclimated to pulse feeding. Correspondingly, the duration of the feast period decreased from 70 to 45 minutes after adaptation to the pulse feeding as also observed from dissolved oxygen profiles (Fig. 4.13b and Figure 4.15a, respectively).



(a) (b)

Figure 4.14: Dynamic behaviour of biomass acclimated to pulse feeding in SBR 8 when applying (a) usual pulse feeding and (b) single disturbance by continuous feeding.



(a) (b)

Figure 4.15: Dissolved oxygen profile of biomass acclimated to pulse feeding in SBR 8 when applying (a) usual pulse feeding and (b) single disturbance by continuous feeding.

This is in agreement with the different length of the feast phase for the different feeding patterns, which the biomass was acclimated to. Indeed, under continuous feeding, the length of feast phase corresponds to the length of feed phase (150 minutes out of 180 minutes of the reaction phase) whereas under pulse feeding, the length of feast phase is quite shorter, as determined from the substrate removal rate after the initial concentration peak.

On the contrary, when the biomass acclimated, pulse feeding was disturbed by using continuous feeding; a continuous decrease in PHA concentration was observed (around 37 mgCOD/L) although the culture was simultaneously using acetate (Figure 4.14b). This behaviour can be explained by considering that the biomass is likely to be acclimated under pulse feeding; hence, its specific substrate uptake rate is high during the feast phase as well as PHA consumption is fast during the famine phase for growth metabolism. Thus, in the presence of slow feeding of acetate, PHA consumption is also activated to maintain the previously higher level of growth metabolism. Accordingly, because acetate partially supports the metabolism, the consumption rate of the storage product during acetate feed is slower than during the last part of the reaction phase after the feed ends up. Çığgın et al. (2007) have reported similar observation for the biomass acclimated in similar conditions under anoxic conditions. In this study, the optimization of the growth mechanism was observed when the carbon source was provided continuously at lower concentration to the activated sludge culture acclimated to pulse feeding. A summary of the biomass response obtained from dynamic experiments is given in Table 4.5.

Short-term effects are shown in Table 4.5 by comparing column 1 versus column 2 and column 3 versus column 4, i.e. the same biomass is experiencing a sudden shift from the usual feeding to the opposite one. It is evident that the substrate removal rate was always higher under pulse feeding than under continuous feeding; accordingly, both storage rate and storage yield were higher under pulse feeding than under continuous feeding. In addition to this observation, a short-term effect due to a single spike was also reported for several microorganisms in pure culture studies where microorganisms had been previously cultured a steady state in a chemostat (Majone and Tandoi 2002).

On the other hand, by comparing columns 2 and 3 with column 1 of Table 4.5, it is clear that the short-term effects of pulse feeding (columns 2 versus 1, single

disturbance on unacclimated biomass) were lower than the long-term effects (column 3 versus 1, 10 days acclimation of the biomass under pulse feeding). Dealing with PHA storage rate, short-term response to single pulse feeding increased the PHA production rate from 16 to 52 mgCOD/gCOD.h, whereas long-term acclimation further increases it to 123 mgCOD/gCOD.h. In other words, when a continuously fed acclimated biomass was shifted to a pulse fed acclimation (all other conditions being the same), the full span of PHA storage rate is really high, from 16 to 123 mgCOD/gCOD.h (107 mgCOD/gCOD.h more, a 769% increase). However, about one third of this span was immediately observed with a single disturbance (36 mgCOD/gCOD.h more, a 325% increase). It is noteworthy that neither full adaptation nor significant change of microbial composition can be supposed to occur in a single disturbance by pulse feeding.

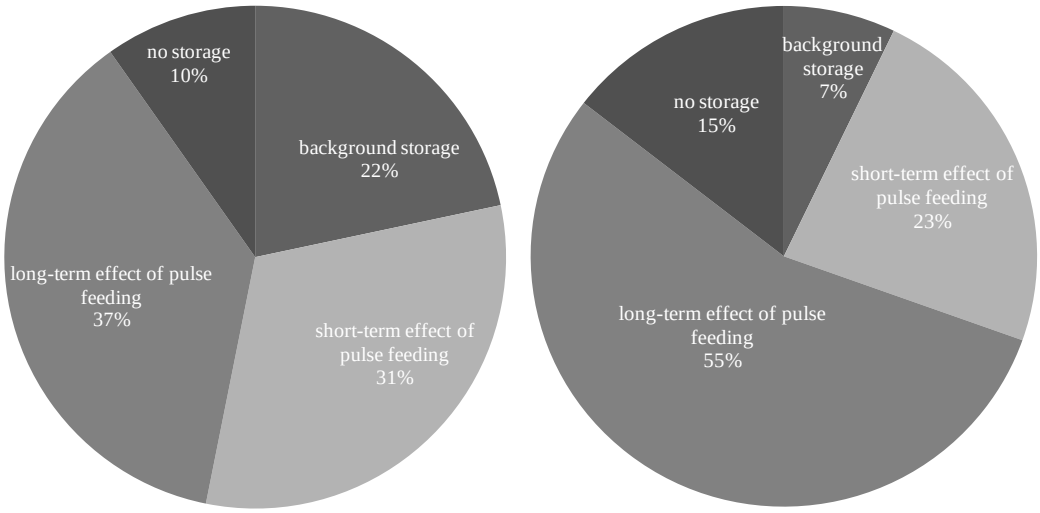
Table 4.5: Dynamic behaviour under different feeding conditions.

	SBR 7		**SBR 8**	
Acclimation Condition	Continuous		Pulse	
Feeding Pattern during dynamic experiments	Continuous	Pulse	Pulse	Continuous
F/M (mgCOD/mgCOD.d)	0.80	0.80	1.04	1.06
Feast Phase length (min)	150	70	45	150
Y_{OBS} (gCOD/gCOD)	0.55	0.52	0.36	0.37
COD fraction used for storage	0.32	0.46	0.54	-
Specific Substrate Uptake Rate, $-q_s$ (mgCOD/gCOD.h)	53	114	232	70
Specific PHA Production Rate, q_p (mgCOD/gCOD.h)	16	52	123	-
$q_p/-q_s$	0.31	0.45	0.53	-

A similar difference between short-term and long-term effects was also observed in storage yield *($q_p/-q_s$)*. A single pulse feeding on unacclimated biomass leads to a short-term increase of PHA storage yield from 0.31 to 0.45 gCOD/gCOD (a 45% increase), whereas long-term acclimation further increases it up to 0.53 gCOD/gCOD (a 71% increase). Even though the overall span for storage ratio was obviously less than for kinetics, it is noteworthy that almost two thirds of overall span was due to the short-term effect, whereas long-term acclimation only brings to one third an overall effect. Similarly, long-term effects have been reported in pure culture studies (Aulenta et al., 2003; Majone et al., 2007) showing that the adaptation of the

microorganism to different dynamic conditions (e.g. frequency of feed as well as hydraulic residence time) also affected its kinetics and yield of PHA storage.

It is also noteworthy that PHA storage remained significant even if biomass was fully acclimated to the continuous feeding. Under less favourable conditions, that caused both slow acetate uptake rate and slow PHA production rate, PHA storage accounted in any case for 31% of COD removal, that can be considered as a "background" level of storage for acetate removal (at least for the investigated experimental conditions). A summary of relative contribution of storage to yield and rate of acetate removal is given in Figure 4.16.



(a) (b)

Figure 4.16: Summary of relative contribution of a) yield and b) rate of storage to respect to overall acetate removal.

Based on results from Table 4.5, storage contribution was corrected with taking into account that a fixed amount of acetate has to be oxidized to provide energy for storage. The overall acetate consumption was calculated as ΔPHA/YSTO where YSTO is the thermodynamic yield of storage on substrate, taken at 0.73 gCOD/gCOD (Van Aalst-van Leeuwen et al., 1997). Because a long-term effect was clearly distinguishable from a short-term effect, the question arises whether the long-term effect is due to slow adaptation of microorganisms, change of consortium microbial composition, or combination thereof.

4.2.3 Molecular characterization by FISH analysis

In order to answer the question whether the shift from continuous to pulse feeding causes a shift in the microbial composition of the aerobic consortium, fluorescence *in situ* hybridization (FISH) was carried out for detection of the main phyla within Bacteria domain for samples from each feeding pattern. For both feeding conditions, Bacteria were nearly 85% of the total DAPI stained cells (EUB338mix/DAPI). In both cases, the *Betaproteobacteria* were detected as most abundant phylum, whereas small amounts of bacterial cells (<5% in total) hybridized with probes specific to *Alphaproteobacteria, Gammaproteobacteria, Actinomycetes*, *Firmicutes, Chloroflexi* and *Cytophaga–Flavobacteria–Bacteroidetes* groups. Therefore, only cell hybridized with the probe specific to *Betaproteobacteria* phylum were quantified as a proportion of EUB338mix-binding cells and DAPI-binding cells.

As the *Thauera/Azoarcus spp.* was identified as main group responsible for the PHA storage within *Betaproteobacteria* in previous studies (Lemos et al., 2008; Serafim et al., 2006), FISH was also performed with probes specific to members of *Thauera/Azoarcus* group (probes AZA645 and THAU832). The analysis showed that the *Thauera/Azoarcus* group comprised nearly 52% and 54% of the total bacteria and almost 57% and 58% of the *Betaproteobacteria* for the biomass acclimated to continuously feeding or pulse feeding, respectively (Figure 4.17). FISH images of cells belonging to *Thauera/Azoarcus* group detected in the inoculum and in the SBRs are shown in Figure 4.18.

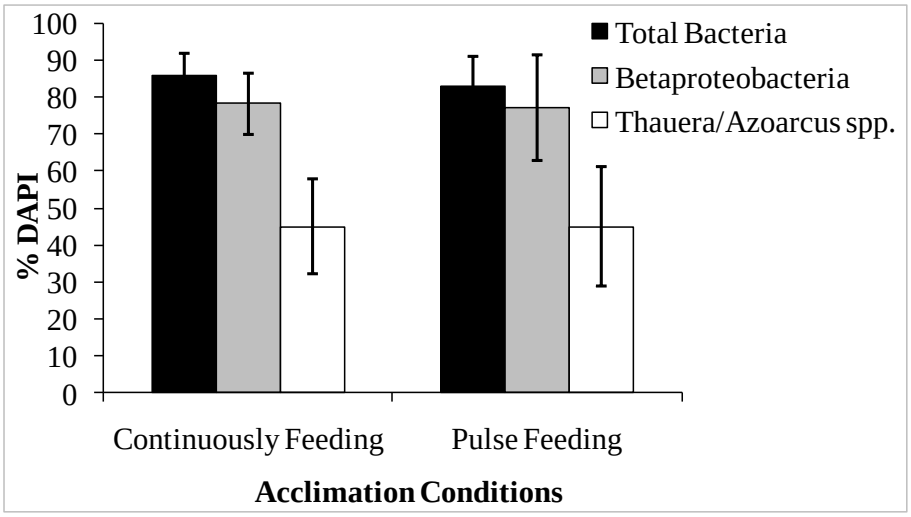
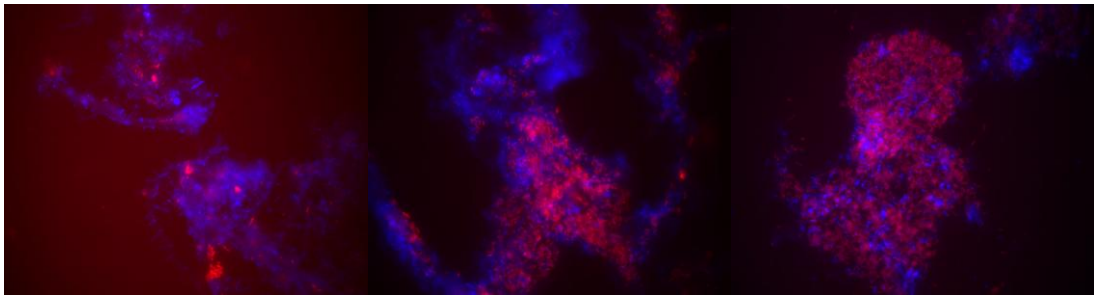


Figure 4.17: FISH quantification of the main microbial components of biomass acclimated to different feeding conditions.

Post-FISH Nile Blue staining was performed on the samples hybridized with *Thauera/Azoarcus* group in order to combine the molecular identification by FISH with the proof of their capability to store intracellular polymers. Post-FISH Nile Blue staining showed that all cells binding to the *Thauera/Azoarcus* probes were involved in PHA storage in pulse fed SBR although some of the *Thauera/Azoarcus*-binding cells contained PHA inclusion in continuously fed system. No Nile-Blue staining was observed on other cells which indicated that the *Thauera/Azoarcus* group was the only one which was responsible for storage.



(a) (b) (c)

Figure 4.18: FISH images of Thauera/Azoarcus-binding cells (red) and DAPI-binding cells (blue) in (a) inoculum, (b) SBR 7 (c) SBR 8.

Although continuous versus pulse feeding conditions caused strong differences in PHA storage rate and yield, no significant differences were observed in terms of microbial composition of respective steady-state acclimated microbial consortia.

Although, no differences in microbial composition were also observed in SBRs 3 and SBR 4, which were acclimated under same operating conditions with SBR 7 and SBR 8 excepting the inoculum, filamentous bacteria belonging to *Alphaproteobacteria* were detected as main responsible for PHA storage in SBR 3 and SBR 4. As the only difference between these two sets of experiments was the inoculum utilized for the start-up of the SBRs, it can be argued that the inoculum played a major role on population dynamics whereas the feeding pattern was not able to alter microbial composition by itself. Thus, in both cases, it was demonstrated that the clear effect of feeding pattern on substrate removal mechanism is mostly due to the adaptation of same microorganisms more than to any shift of microbial composition.

5. EFFECT OF FEEDING PATTERN AND SLUDGE AGE ON BACTERIAL COMMUNITY AND FATE OF SLOWLY BIODEGRADABLE SUBSTRATE

5.1 The Effect of Feeding Pattern on the Starch Removal Mechanism

The effect of pulse or continuous feeding on substrate utilization mechanism was first investigated in the SBR unit operated at a sludge age of 8 days. Characteristics related to the steady-state performance of the SBRs are summarized in Table 5.1. The pulse feeding at the beginning of each cycle was adjusted to induce an initial starch concentration of 203 mgCOD/L and the SBR unit sustained an average biomass concentration of around 2000 mg VSS/L at steady state with a glycogen pool of 416 mg COD/L, corresponding to around 15% of the biomass on a COD basis.

Table 5.1: Steady state performance of the SBRs.

Parameter	SBR 9	SBR 10	SBR 11	SBR 12
Experimental SRT (1/d)	8.04	8.06	2.07	2.05
Substrate Concentration (mgCOD/L.cycle)	203	230	267	287
Average VSS (mgVSS/L)	2000 ± 16	1790 ± 18	1300 ± 10	1230 ± 10
Y_{OBS} (gCOD/gCOD)	0.29	0.23	0.56	0.49
Feast Phase (min)	10	150	15	150
Glycogen Pool (mgCOD/L)	416	156	282	172
Amount of Stored Glycogen (mgCOD/L.cycle)	170	86	213	99
COD fraction used for storage	0.84	0.37	0.80	0.34
COD Removal (%)	92	90	90	81

As shown in Figure 5.1a, starch – measured as glucose – could only be detected as 42 mg/L due to sampling frequency in the very short time period and it was depleted within the first 3-4 minutes. The observable soluble COD with pulse feeding was 105 mg/L; it was reduced within a relatively longer period of 10 minutes, down to 33 mg/L, the baseline COD level initially present in the idle phase and sustained throughout the cycle. This level should not be related to starch which is a

biodegradable substrate and therefore totally removed, but to residual soluble microbial products generated as part of the microbial activity (Orhon et al., 1999).

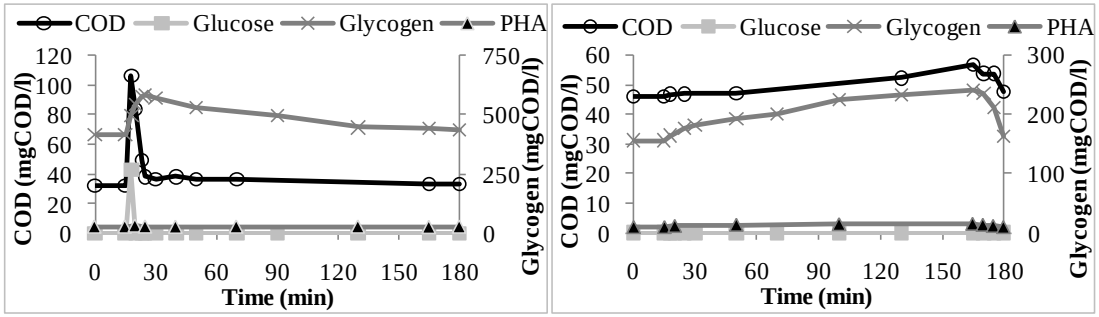


Figure 5.1: Observed concentration profiles of soluble COD, starch, glycogen and PHA (a) with pulse feeding and (b) with continuous feeding in a cycle of SBR at sludge age of 8 days.

Glycogen storage started with pulse feeding and reached its peak level after ten minutes of the reaction phase with an incremental glycogen generation of 170 mgCOD/L and gradually reduced afterwards as internal substrate. Measurements also indicated a much lower PHA pool of 28 mgCOD/L but no additional PHA generation with pulse feeding (Figure 5.1a). Continuous starch feeding in the parallel SBR operation also sustained internal substrate storage, although with a much lower average glycogen pool of 156 mgCOD/L, which also reduced the biomass concentration to around 1800 mgVSS/L at steady state. The glycogen generation was limited to 86 mg COD/L, half the level associated with pulse feeding. Consequently, the level of glycogen storage remained limited to 37% of starch supplied during the cycle. The internal storage process continued throughout the end of the feeding period while no starch concentration was detected in the reactor (Figure 5.1a). The corresponding soluble COD level was slightly higher (46 mg/L) and exhibited only a gradual increase to 57 mg/L at the end of the feeding period. PHA generation was still not significant during cyclic operation (Figure 5.1b). As shown in Figure 5.2b, the corresponding DO profile was quite different as compared with the one obtained for pulse feeding and exhibited a steady level around 90% of the saturation level during substrate feeding.

Changes in the dissolved oxygen (DO) concentration under constant aeration regime and the corresponding oxygen uptake rate (OUR) profile were also monitored. As shown in Figure 5.2a, the DO profile exhibited a decrease from 70% to around 25%

within the first ten minutes, parallel to glycogen storage, and it was gradually increased during the remaining part of the reaction phase.

(a) (b)

Figure 5.2: Observed dissolved oxygen profiles during a cycle of (a) pulse fed SBRs and (b) continuous fed SBRs.

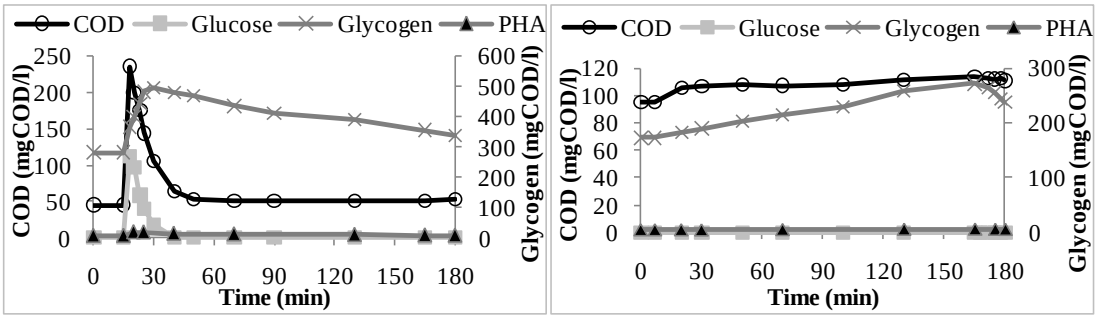
The observed results for pulse feeding indicate a typical feast and famine cycle behaviour. During the feast phase, 84% of external substrate was converted to glycogen. The DO concentration decreased until and the glycogen concentration reached its maximum level in 10 minutes. During the following famine phase biomass activity solely depended upon utilization of internally stored substrate.

5.2 Effect of Sludge Age on the Starch Removal Mechanism

This part of the experimental study involved two parallel SBR units started with fresh biomass seeding from the same treatment works. The units were subjected to the same pulse and continuous feeding regimes as previously and they were acclimated for steady state operation at a sludge age of 2 days substantially different from the first part, to visualize the effect of sludge age on starch utilization. The same cyclic operation was maintained but the level of cyclic starch feeding was increased around 30%, as indicated in Table 5.1, to obtain relatively higher biomass concentrations at low sludge age. The average behaviour of the SBR units in terms of the COD removal and glycogen production are given in Figure 5.3.

As plotted in Figure 5.3 and summarized in Table 5.1, the substrate utilization pattern basically remained the same as in SBR runs previously conducted at a higher sludge age of 8 days. In other words, the results indicated that sludge age did not appear to be a significant factor in changing the substrate the utilization mechanism of starch, based on following observations: (i) SBR operation at steady state still

generated a glycogen pool defined by specific process kinetics and mass balance. (ii) Under pulse feeding, major portion of starch was internally stored as glycogen; the average amount of stored glycogen in each cycle was 213 mg COD/L corresponding to 80% of the available substrate COD. (ii) Under continuous feeding, glycogen storage was reduced to 99 mg COD/L, i.e. 34% of the starch COD supplied during the cycle.



(a) (b)

Figure 5.3: Observed concentration profiles of soluble COD, starch, glycogen and PHA (a) with pulse feeding and (b) with continuous feeding in a cycle of SBR at sludge age of 2 days.

As given in Table 5.1, relatively similar amount of the carbon source was used for the storage in the SBRs acclimated to a sludge age of 2 days. The interesting feature of pulse feeding was that periods of COD removal; starch depletion, glycogen and generation oxygen drop overlapped with each other, indicating the synchronous nature of these processes (Figure 5.2a and Figure 5.3a). This is quite different from SBR operation at a higher sludge age with the same feeding regime where starch removal occurred almost immediately with a significant time difference with the completion of glycogen formation.

5.3 Molecular Characterization by FISH

After the establishment of the steady state operating conditions, the bacterial composition of each SBR was determined by FISH. Most of Bacteria were identified by oligonucleic probes specific for the main phyla within Bacteria domain and their distribution in each run is given in Figure 5.4. *Actinobacteria* were found as the dominant phylum (accounting for 40-50% of the total bacteria) in most of the analysed samples (SBRs 9-11). In addition to this group, *Gammaproteobacteria* and

Betaproteobacteria were also detected in all SBRs. The distribution of the filamentous bacteria in SBRs is given in Figure 5.5.

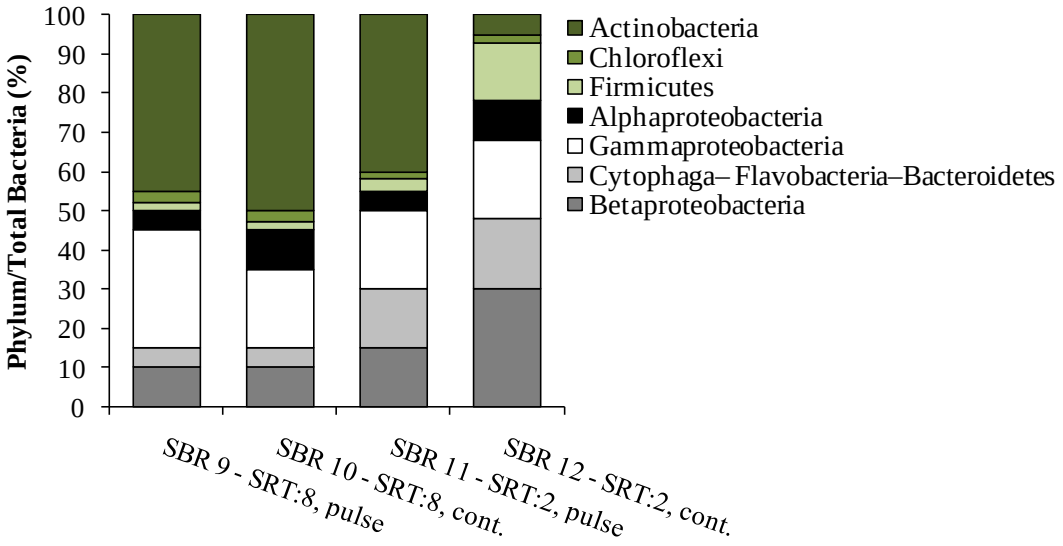


Figure 5.4: Bacterial population dynamics estimated by FISH analysis as a percentage of total bacteria in each SBR.

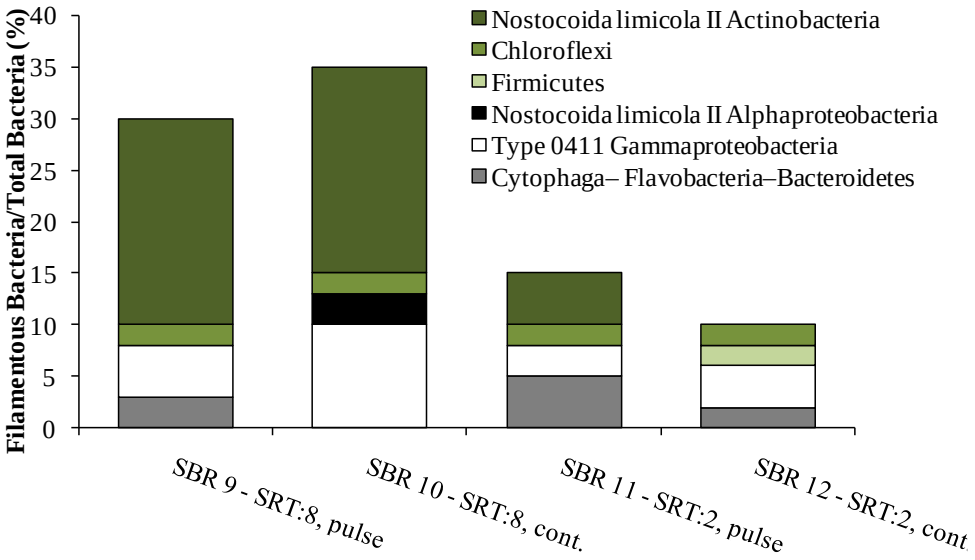
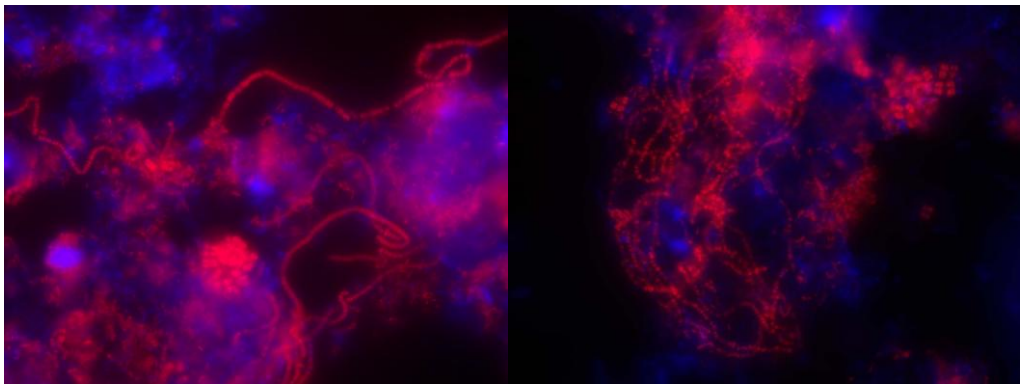


Figure 5.5: Amount of filamentous bacteria estimated by FISH analysis as a percentage of total bacteria in each SBR.

In SBRs operated at higher SRT (SBR 9 and SBR 10) almost one third of the bacterial population was detected as filamentous. The dominant filamentous bacteria belonging to *Actinobacteria* found in SBRs 9 and 10 were identified as *Nostocoida limicola* as seen in Figure 5.6. In spite of the presence of considerable amounts of filamentous bacteria, no bulking events occurred during the SBRs operation.



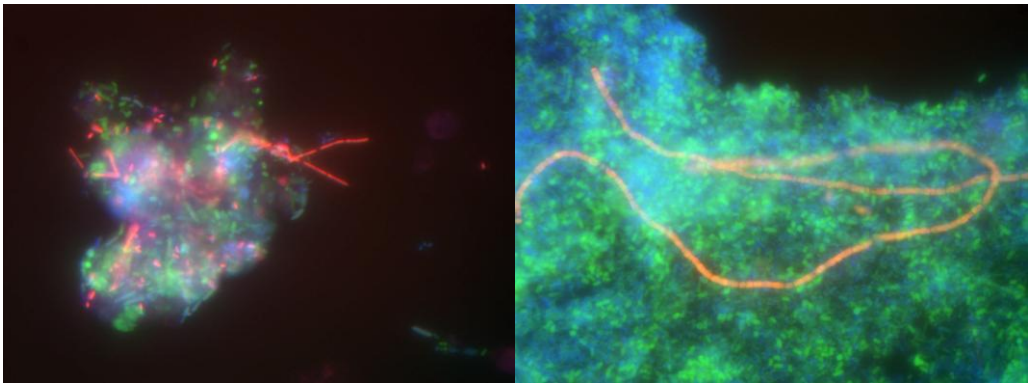
(a) (b)

Figure 5.6: FISH Results for (a) SBR 9 and (b) SBR 10; DAPI (blue) and HGC (red).

Although high amount of the filamentous bacteria were detected in the SBRs operated at the SRT of 8 days, the observation of the well settling sludge (SVI<100 mL/g) has been supporting the observation of Martins et al. (2004). This author reported that sludge bulking was never experienced in the experiments performed with starch independently from the feeding pattern or oxygen concentration. This observation has been explained with the uniformly distribution of the hydrolysis products inside the flocs. When substrate is taken up at low concentrations from the bulk solution this would lead to gradients over the floc giving advantage to filamentous organisms which extend outside the floc (Martins et al., 2010).

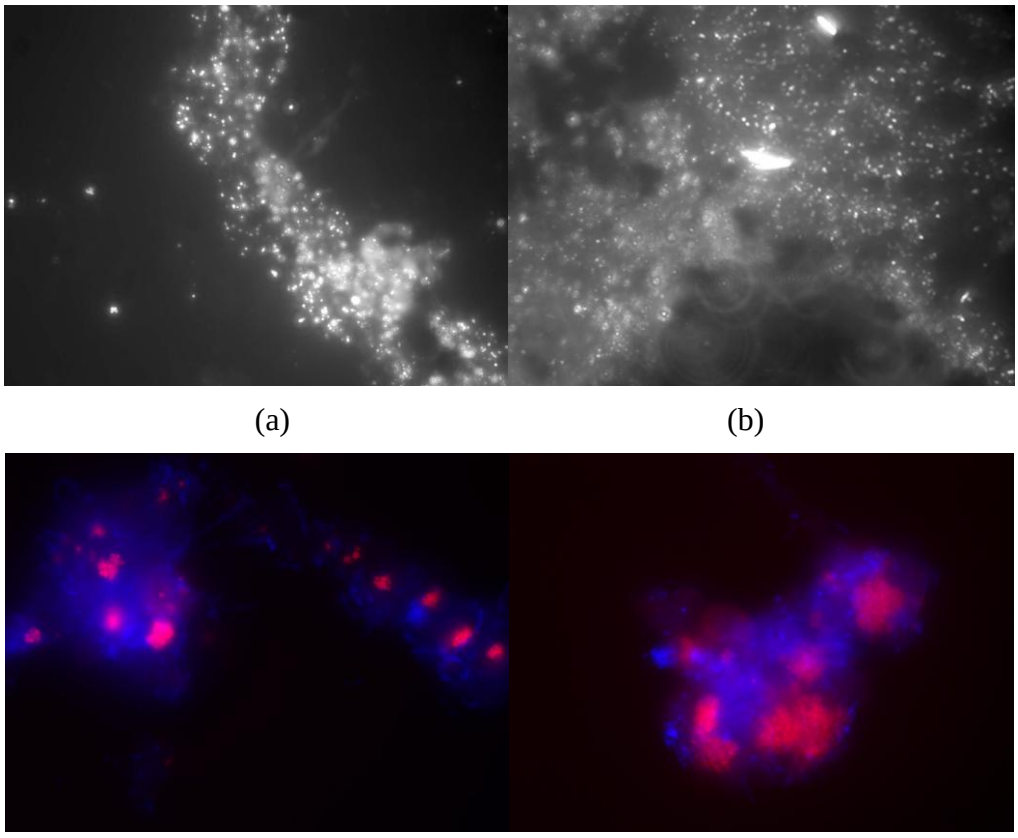
The most abundant filamentous bacteria were detected as belonging to *Chloroflexi* and *Cytophaga–Flavobacteria–Bacteroidetes* groups in SBR 11 and SBR 12, respectively; the representative pictures of these two groups are shown in Figure 5.7.

As the Nile Blue staining showed the PHA inclusions in SBRs 11 and 12 operated at SRT of 2 days, the analysis was refined by applying probes specific for members of Thauera/Azoarcus group (probes AZA645 and THAU832) which were previously reported as responsible for PHA storage in periodic systems (Dionisi et al., 2005). Almost all Betaproteobacteria in SBRs operated at SRT of 2 days were further identified as members of Thauera/Azoarcus group. The representative Nile Blue staining image and FISH pictures for the SBRs operated at the SRT of 2 days are given in Figure 5.8.



(a) (b)

Figure 5.7: The filamentous bacteria detected in (a) SBR 11 belonging to *Citophase/Flexibacter/Bacteroides* (red) and (b) SBR 12 belonging to *Chloroflexi sp.* (red), DAPI (blue) EUBmix (green).



(a) (b)

(c) (d)

Figure 5.8: Intracellular PHA inclusions (a and b) after Nile Blue staining (a) Set 11, (b) Set 12; and the photomicrographs of FISH (c and d) with AZA645/THAU832 probe (red) and DAPI (blue) (c) Set 11, (d) Set 12.

Bacterial composition sustained under different operating conditions should also be considered as a likely parameter to exert an influence on the selection of substrate removal mechanism. In this context, results of FISH analyses indicated the following points. Biomass composition under pulse feeding remained essentially the same, with

A*ctinobacteria* as the major bacterial group. This group was also reported as the starch hydrolyzing organisms by starch staining combined with FISH analysis, in the study carried out with samples taken from several wastewater treatment plants (Xia et al. 2008). In that study, it was also suggested that filamentous microorganisms acted as consumers of starch hydrolysates, because inhibitor addition to eliminate the consumers of starch hydrolysates only decreased the amount of filamentous biomass.

At high sludge age, continuous feeding did not significantly alter biomass composition; but at low sludge age, it affected the bacterial community established under selected operating conditions. Biomass sustained at two different sludge age always included bacterial fractions that could utilize starch for internal storage of glycogen. Sludge inflicted a significant change on the relative extend of filamentous microorganisms: Low growth rate conditions at high sludge operation favoured filamentous growth. This observation can probably be explained by means of the kinetic selection theory (Chudoba et al., 1973). According to this theory, when the substrate is available in low concentration, filamentous bacteria become competitively advantageous. As high molecular weight substrates like starch were enzymatically hydrolyzed into biomass before becoming available for utilization by the bacterial community - either through growth and/or storage - hydrolysis step becomes the rate limiting step for substrate removal (Gujer et al., 2000). Consequently, consumable substrate will be available for the microorganisms at low (growth rate limiting) concentrations when the activated sludge is fed with high molecular weight substrate (Martins et al., 2010). Conversely, competitive advantage of the floc formers in the SBRs operated at a sludge age of 2 days may be explained with the fact that complete renewal period of the microbial culture in the reactor is much shorter, i.e. determined by sludge age; thus a low sludge age results in a much faster growth rate for this renewal to occur (Katipoglu et al., 2010).

Despite filamentous growth, no bulking was observed. Despite high levels of filamentous microorganisms detected in the SBRs operated at a sludge age of 8 days, the observation of the well settling sludge (SVI<100 mL/g) was consistent with the results of previous studies: Ovez and Orhon (2005) provided experimental proof that bulking depended on the occurrence and relative distribution of specific filamentous microorganisms. Martins et al. (2004) have reported that they have never experienced sludge bulking independently from the feeding pattern or oxygen

concentration during the experiments with starch. This observation was explained by the uniform distribution of the hydrolysis products inside the floc. When substrate was taken up at low concentrations form the bulk solution, this would lead to gradients within the floc giving competitive advantage to filamentous organisms which extended outside the floc (Martins et al., 2010). Similarly, one of the dominant filamentous microorganisms - Type 0411 - detected in abundance in activated sludge systems, was found to exert no adverse effect on the settling properties of the biomass (Guo et al., 2010).

5.4 Comparison of the PHB and Glycogen as Storage Polymers

The experimental results indicated the feeding regime as the major parameter dictating the specific substrate utilization mechanisms; the sludge age appeared to only have secondary effect on this mechanism: Collected data on substrate removal, glycogen production and oxygen utilization profiles all point out that the rapid uptake and storage of the starch as glycogen were the prevalent behaviour of the biomass acclimated to pulse feeding. Continuous feeding substantially lowered glycogen formation and diverted a part of substrate utilization to direct microbial growth independently from the selected sludge age. Consequently, simultaneous storage and direct growth on primary substrate was the observed mechanism of starch utilization under continuous feeding (Krishna and Van Loosdrecht, 1999a; Karahan-Gül et al., 2003). This observation was also supported with the average rates and ratios calculated from the cycle experiments (Table 5.2).

Table 5.2: Average rates and yields observed in cycle experiments.

Parameter	**SBR 9**	**SBR 10**	**SBR 11**	**SBR 12**
Substrate Uptake Rate, $-q_s$ (mgCOD/gCOD.h)	426	36	579	66
Glycogen Production Rate, q_G (mgCOD/gCOD.h)	355	13	460	22
Glycogen Consumption Rate, $-q_G$ (mgCOD/gCOD.h)	20	122	33	79
$q_G/-q_s$	0.83	0.36	0.80	0.33

The storage ratio for starch calculated from the fraction of removal substrate to produced storage polymer ($q_G/-q_S$) was found as nearly 0.80 – 0.83 gCOD/gCOD for pulse fed SBRs and 0.33 – 0.36 g COD/gCOD for continuously fed SBRs independently from the sludge age. The range of glycogen storage in pulse fed SBRs

confirms the previously reported storage capacities with starch, namely 75% (Karahan et al., 2005) and almost 80% (Martins et al., 2010). In the earlier phase of the study conducted with acetate – a simple, readily biodegradable substrate – the sludge age induced a decreasing effect on the PHA storage when it was lowered to 2 days; this change could be explained with a corresponding increase in the portion of acetate consumed directly for growth process, probably because of the higher maximum growth rate obtained at a sludge age of 2 days (Çığgın et al., 2011). The more energy-efficient storage of glycogen as compared to PHA compounds should also be envisaged as another factor in this observation (Dircks et al., 2001).

In the pulse-fed SBR operated at a sludge age of 8 days (SBR 9), 83% of the available substrate was converted to glycogen; this ratio is very close to reported yield values for storage, Y_{STO}: in fact, a range Y_{STO} values of 0.87 – 0.93 g COD/g COD were suggested for glucose and starch (Goel et al, 1998; Dirks et al., 2001; Karahan-Gül et al. 2003) higher than the level 0.80 g COD/g COD) adopted as a default value in ASM3 (Gujer et al., 2000) because glycogen storage is energetically more efficient as compared to PHB. In this case, starch was almost entirely used for storage. Higher growth response at the sludge age of 2 days only resulted in a slight decrease in the glycogen ratio to 80%, with a slight diversion of substrate utilization for direct growth.

The other issue which may be interpreted as a likely impact of the sludge age on substrate utilization is the occurrence of a preliminary adsorption process in starch removal: the time difference between the rapid uptake of starch from solution and other processes in the feast phase, i.e. glycogen generation, DO drop, etc signal the presence of initial starch adsorption. A similar finding was also reported by Karahan et al. (2005) and later confirmed by model evaluation (Karahan et al., 2006). At sludge age of 2 days however, starch was not consumed so quickly: It was simultaneously depleted at the end of feast phase which coincided with the consumption of all influent COD, glycogen formation and decrease in the oxygen utilization. This leads to suggest preferential direct utilization of the starch without adsorption by the microorganisms having a higher growth rate in SBR operation with lower sludge age.

6. MODELLING OF DYNAMIC BEHAVIOR IN ACTIVATED SLUDGE

6.1 Modelling of Kinetic and Stoichiometry for Readily Biodegradable Substrate Removal in SBRs

6.1.1 Model structure

The model adopted for this study involved the basic template of ASMG – *Activated Sludge Model for Growth and Storage* - previously suggested by Krishna and van Loosdrecht (1999a) and implemented for evaluation the utilization mechanism of starch (Karahan et al., 2006). It included a combination of relevant components and processes of ASM1 and ASM3 necessary for removal of acetate, a simple, readily biodegradable substrate. The model had six components, namely, concentrations of acetate, S_{AC}, stored internal PHB, X_{PHB}, active heterotrophic biomass, X_H, soluble and particulate residual microbial products, S_P; X_P and finally dissolved oxygen, S_O. Accordingly, it incorporated four biochemical processes: direct growth on S_{AC}; storage of S_{AC} as X_{PHB}; secondary growth on X_{PHB}; endogenous respiration of X_H. Respiration of X_{PHB} as suggested in ASM3 was also added as the fifth process to be compatible with the template ASM3 structure. Generation of microbial products, S_P and X_P were implicitly included as part of endogenous respiration with the simplifying assumption of decay-associated processes adopted in many similar studies. Related rate expressions and basic stoichiometry were defined in a way compatible with previous mathematical models. Switching functions of ammonia nitrogen, dissolved oxygen and alkalinity were omitted in the rate expressions, because they were maintained in excess of rate limiting levels in the SBR reactor fed with the synthetic substrate feed solution.

Model calibration and evaluation were conducted by means of the AQUASIM simulation program (Reichert et al., 1994). Manual calibration of model components started each iteration step, fitting all model outputs on real time data. The model outputs were equally sensitive to all relevant model coefficients. The calibration essentially involved two consecutive steps: First, a steady-state calibration using the average volatile suspended solids (MLVSS) data of the SBR system, in order to

secure solids mass balance by setting the endogenous respiration coefficients. Then, the model was calibrated to determine the stoichiometric and kinetic parameters of growth and storage processes using dynamic simulation of the concentration profiles within the representative cycles of SBR at steady state.

6.1.2 Parameter identifiability analysis

The application of identifiability analysis on SBRs operated under different feeding conditions allows the comparison of changes in selected model parameters k_{STO}, K_{S1}, K_{S2}, K_{STO}, μ_{H1}. The reason is that the complete set of parameters was determined to be non-identifiable with the in-cycle experimental OUR, COD and PHB profiles obtained in this study.

In order to tackle this problem, Lukasse et al. (1996) previously underlined the advantage of reducing the number of model parameters in the calibration procedure; they suggested to assign appropriate values to some of them for improving the reliability of estimation and increasing the accuracy of the remaining – more important – parameters. In this context, values of parameters related to endogenous respiration, b_H, b_{STO} and f_{ES}, f_{EX} were estimated as part of the steady state calibration and mass balance over volatile suspended solids (MLVSS); Y_{STO} and Y_{H1} were determined using prescribed methodology based on the evaluation of the OUR curves, as defined in the following section; Y_{H2} was primarily verified with PHB profiles; f_{ES} values were defined from long term COD data. Similar yield coefficients (Y_{H1}) were adopted for direct growth on S_S and growth on PHB processes. This way, the calibration was narrowed down to the estimation of model parameters related to growth on acetate (μ_{H1}, K_{S1}); storage on acetate (k_{STO}, K_{STO}) and growth on stored PHB (μ_{H2}, K_{S2}). Validity of previously determined and fitted parameters was verified and confirmed as part of the model calibration with an additional detailed parameter identifiability study.

The method suggested by Brun et al. (2001) was used to assess identifiability analysis for the selected parameters. As a first step, the parameter importance was determined by ranking the parameters by a sensitivity measure in a decreasing order. After the determination of the suitable parameter subsets for identifiability analysis the collinearity index (γ_K) are calculated based on the sensitivity functions obtained for the parameters with respect to OUR and PHB simulations. The γ_K of a selected

parameter subset has been used for the determination of the sensitivity of an observed model output to small changes of all parameters in the subset on an individual basis. The critical values for γ_K have been suggested as a maximum value of 20. The practical explanation of collinearity index (γ_K) 20 means that a change of the calculated results caused by a shift of a parameter can be compensated by 5% changes in other parameters in the subset. The model simulations and sensitivity analyses were determined with AQUASIM (Reichert et al., 1994). The UNCSIM module (Brun et al., 2001) was used for collinearity index calculations.

6.1.3 Process stoichiometry

Respirometry serves now as an integral component of the modelling procedure. The OUR profile may be used to determine the magnitude of the yield coefficient, the major parameter of process stoichiometry, if the test is started with a known/predetermined amount of substrate. In fact, the OUR area above the endogenous respiration level directly indicates the amount of oxygen, ΔS_O, consumed during substrate utilization. Provided that substrate is totally depleted during the experiment, the equivalence between ΔS_O and the total biodegradable COD, C_S, is set by the yield coefficient, Y_H:

$$\Delta S_O = (1 - Y_H) C_S \qquad \textbf{(6.1)}$$

This expression becomes operative for a given substrate utilization mechanism: If C_S is totally utilized for microbial growth, it defines the heterotrophic yield coefficient, Y_H (Sollfrank and Gujer, 1991). Conversely, if the OUR curve results from total conversion of C_S to internal storage biopolymers, it is used to define the storage yield, Y_{STO} (Karahan-Gül et al., 2002). When the OUR profile reflects the joint impact of simultaneous direct growth and storage, as observed in this study and outlined in the previous section, the above expression becomes:

$$\Delta S_O = (1 - Y_{STO}) S_{AC,STO} + (1 - Y_H) S_{AC,GR} \qquad \textbf{(6.2)}$$

where, $S_{AC,\,STO}$ is the acetate concentration utilized for storage, and $S_{AC,GR}$, for direct microbial growth. A model simulation of the experimental OUR curve obtained in the study is presented in Figure 6.1, indicating the portions of the curve – respective O_2 consumptions – associated with storage/growth (ΔS_O), secondary growth on internal storage biopolymers ($\Delta S_{O,\,GR2}$) and endogenous respiration ($\Delta S_{O,\,ER}$).

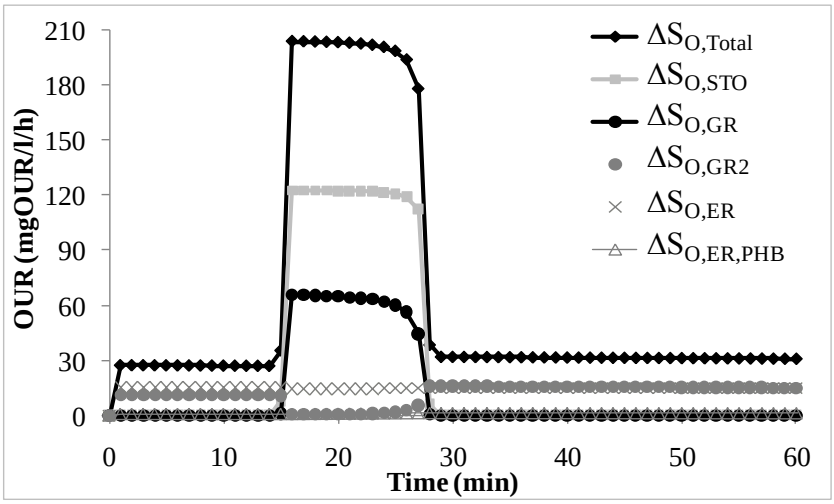


Figure 6.1: Evaluation of the OUR profile according to simultaneous storage and direct growth mechanism.

This expression was applied to the experimental OUR profiles obtained in four different SBR runs, each characterized by different feeding regimes and sludge age values, as outlined in Table 4.2. A successive iteration method was used for assessing the appropriate value of Y_{STO} and Y_H satisfy different oxygen/substrate balances for all the runs. The starting point of the iteration was the observed X_{PHB}/S_{AC} ratios together with assumed initial values for Y_{STO} and Y_H. The evaluation yielded a Y_H of 0.67 mgcellCOD/mgCOD and a Y_{STO} of 0.84 mgCOD/mgCOD with an average variation of %1.3, for defining the process stoichiometry in the model calibration with the experimental data: Y_H = 0.67 mgcellCOD/mgCOD falls within the narrow range of 0.64-0.68 mgcellCOD/mgCOD reported for a wide range of different wastewaters (Orhon et al., 2009); Y_{STO} = 0.84 mgCOD/mgCOD agrees well with the level of 0.80-0.84 mgCOD/mgCOD reported in many similar studies conducted with acetate (Koch et al., 2000; Beccari et al., 2002; Karahan-Gül et al. 2002) and the default value of 0.85 mgCOD/mgCOD in ASM3 (Gujer et al., 2000). It is slightly higher than the range of 0.73-0.82 mgCOD/mgCOD similarly suggested for acetate (van Aalst-van Leuven et al., 1997; Karahan-Gül et al., 2002).

6.1.4 Modelling of process kinetics

Evaluation of system behaviour and performance using the adopted model was performed on all four SBR runs operated at two different sludge ages of 8 and 2 days and under pulse and continuous feeding regimes. The model was calibrated using the cyclic OUR curves and the concentration profiles of principal organic carbon components, i.e. COD and PHB profiles. The adopted model included, as given in

Table 6.1, stoichiometric and kinetic parameters defining related rate expressions of five different processes.

Table 6.1: Matrix representation of proposed model for Readily Biodegradable COD (S_{AC}).

Component / Process	S_{AC}	X_{PHB}	X_H	S_P	X_P	S_O	Process rate
Growth on S_{AC}	$-\frac{1}{Y_H}$		1			$-\frac{1-Y_H}{Y_H}$	$\mu_{H1}.\frac{S_{AC}}{K_{S1}+S_{AC}}.X_H$
Storage of S_{AC}	-1	Y_{STO}				$-(1-Y_{STO})$	$k_{STO}.\frac{S_{AC}}{K_{S2}+S_{AC}}.X_H$
Growth on PHB		$-\frac{1}{Y_H}$	1			$-\frac{1-Y_H}{Y_H}$	$\mu_{H2}.\frac{K_{S1}}{K_{S1}+S_{AC}}.\frac{X_{PHB}/X_H}{K_{STO}+X_{PHB}/X_H}X_H$
Endogenous respiration			-1	f_{ES}	f_{EX}	$-(1-f_{ES}-f_{EX})$	$b_H.X_H$
Endogenous Respiration of X_{PHB}		-1				-1	$b_{STO}.X_{PHB}$

6.1.5 Result of the identifiability analysis

The identifiability analysis was conducted for evaluating the main kinetic parameters, namely μ_{H1}, K_{S1}, $K_{S2,}$ k_{STO}, $K_{STO,}$ and in accordance with the method proposed by Brun et al. (2001). In order to get information on the identifiability of parameter subsets, collinearity indices were calculated for parameter subsets. Table 6.2 summarizes the collinearity measures of selected parameters obtained in the identifiability study. Based on the results given in the Table 6.2, the subset including 4 parameters namely k_{STO}, K_{S1}, K_{S2}, μ_{H1} or k_{STO}, K_{S1}, K_{S2}, K_{STO} were found to be identifiable by fulfilling the suggested conditions as γ_K<20 (Brun et al., 2001). It should be noted that the difficulty in estimating μ_{H1} and K_{STO} parameters together is due to the kinetic structure given in "Growth on PHB" process. The co-existence of those parameters as multiplier and divider in the same expression results in high correlation which was also governed by a high collinearity level of 59 as indicated in Table 6.2. However, four (4) of important kinetic parameters were found to be identifiable. The output seems to be sufficient for the accurate implementation of calibration because the other parameters were either identified by solids mass balance or by process stoichiometry, as previously explained. The identifiability

analysis served as a basis to compare the differences in important parameters in pulse and continuous fed SBR systems.

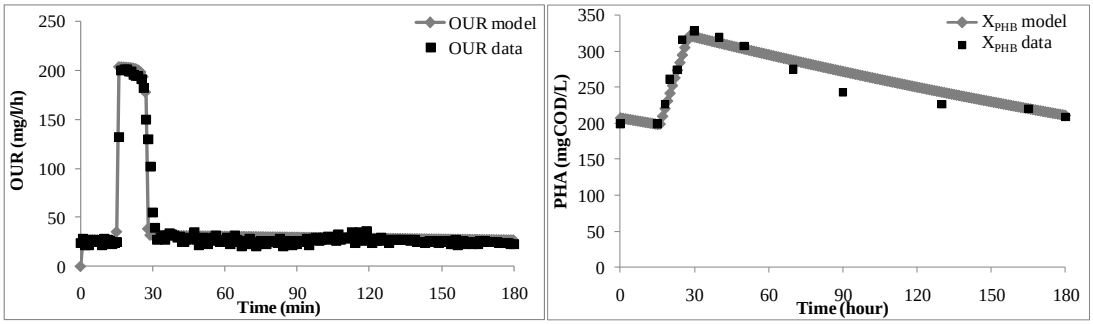
Table 6.2: Collinearity Indices (γ_K) for subsets SBRs.

	Parameter Subsets		
	#1	**#2**	**#3**
Parameters	k_{STO}	k_{STO}	k_{STO}
	μ_{H1}	K_{STO}	μ_{H1}
	K_{S1}	K_{S1}	K_{S1}
	K_{S2}	K_{S2}	K_{S2}
			K_{STO}
γ_K	**8**	**7**	**59**

The values of the calibrated model parameters were summarized in Table 6.3. The results, outlined in Table 6.3, support simultaneous growth and storage mechanisms for the utilization of acetate and define corresponding values for the model coefficients incorporated in related process rate expressions. The graphical representation of the model calibration exercise carried out for all the SBR runs operated at steady state under different conditions are illustrated in Figures 6.2 - 6.5.

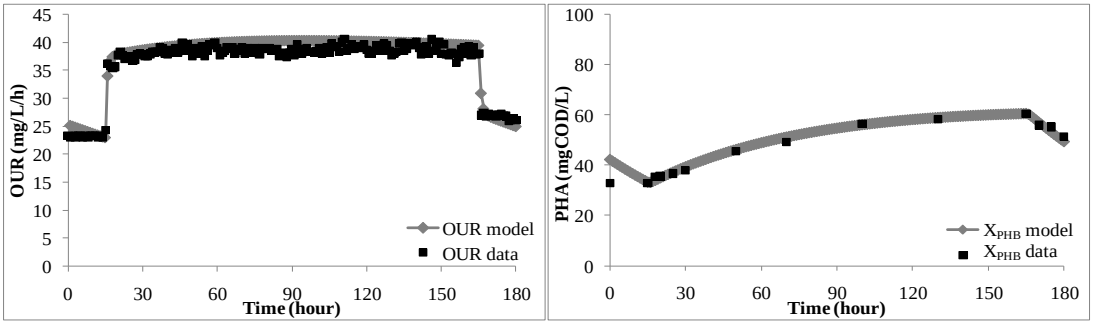
Table 6.3: Results of model calibration for SBRs.

Parameter	**Units**	**SBR 1**	**SBR 2**	**SBR 3**	**SBR 4**
		Pulse	**Cont.**	**Pulse**	**Cont.**
		sludge age =8 days		**sludge age =2 days**	
Y_{H1}	gcellCOD/gCOD	0.68	0.68	0.68	0.68
Y_{STO}	gCOD/gCOD	0.84	0.84	0.84	0.84
μ_H	1/d	1.5	1.5	2.5	2.5
k_{STO}	1/d	8.0	8.0	6.5	6.5
K_{STO}	gX_{PHB}/gX_H	0.45	0.07	0.50	0.23
K_{S1}	gCOD/L	5	25	10	25
K_{S2}	gCOD/L	1	1	1	1
b_H	1/d	0.20	0.20	0.20	0.20
b_{STO}	1/d	0.10	0.10	0.10	0.10
f_{ES}	-	0.05	0.05	0.05	0.05
f_{EX}	-	0.10	0.10	0.10	0.10



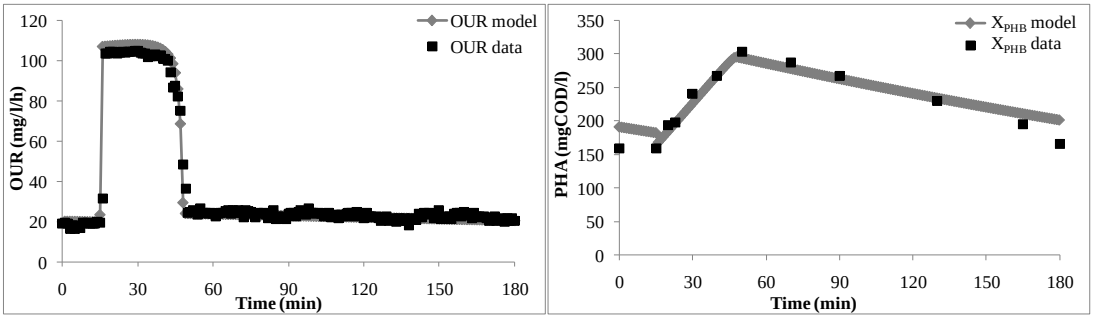
(a) (b)

Figure 6.2: Simulation Results of SBR 1 for (a) OUR, (b) PHB.



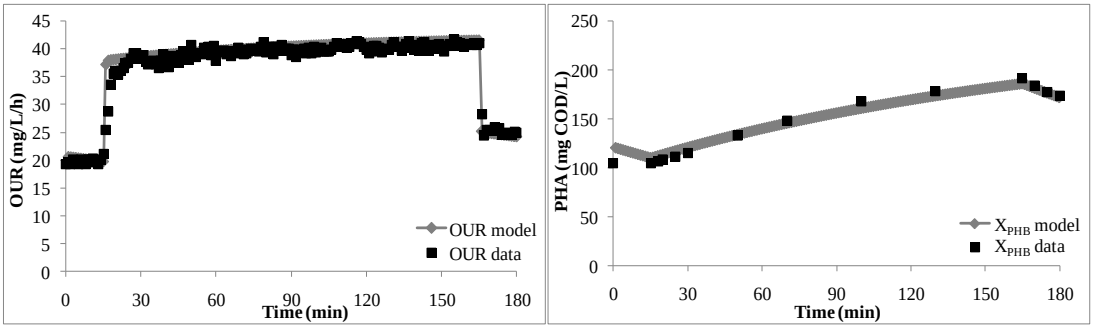
(a) (b)

Figure 6.3: Simulation Results of SBR 2 for (a) OUR, (b) PHB.



(a) (b)

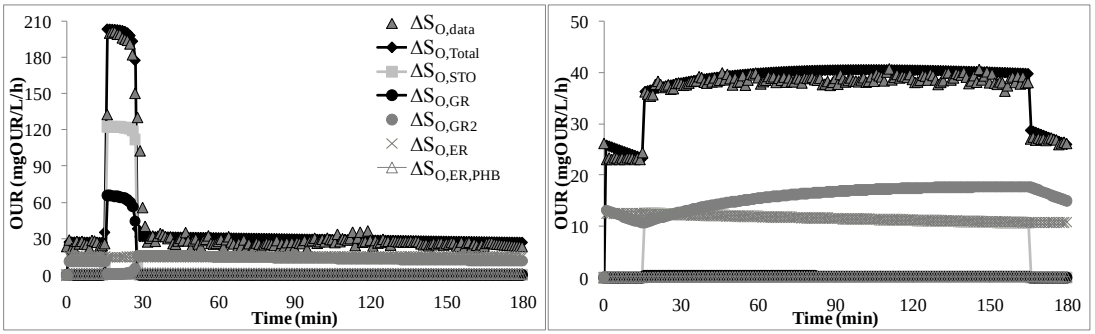
Figure 6.4: Simulation Results of SBR 3 for (a) OUR, (b) PHB.



(a) (b)

Figure 6.5: Simulation Results of SBR 4 for (a) OUR, (b) PHB.

The figures show the close fit obtained between the experimental data and the model simulation using the calibrated model coefficients; they also indicate that the model remains equally applicable to OUR and PHB profiles reflecting different feeding regimes and culture history. For SBR operation with pulse and continuous feeding at a sludge age of 8 days, a model simulation is also included to reflect the relative magnitude of the OUR data associated with direct growth and storage (Figure 6.6). The main observation derived from the results was that feeding regime and culture history – i.e. sludge age – exerted different impacts on process kinetics. While the effect of sludge age was observed on the rate parameters of storage and direct growth (k_{STO}; μ_{H1}); the feeding regime mainly affected the corresponding half saturation coefficients (K_{STO}; K_{S1}).



(a) (b)

Figure 6.6: OUR Fraction of (a) pulse and, (b) continuously fed SBR at the sludge age of 8 days.

The change in the sludge age from 8 days to 2 days slightly reduced the k_{STO} value from 8.0 1/day to 6.5 1/day and increased the corresponding μ_{H1} value from 1.5 1/day to 2.5 1/day, regardless of the feeding regime implemented. Although the differences are small, they suggest and support that microbial consortium can adjust their the protein synthesis systems to accommodate higher levels of external substrate for growth when it is adapted to faster growth conditions (lower sludge age) as opposed to biomass adjusted to lower growth conditions (higher sludge age) where a higher fraction of external substrate is diverted to internal storage (Daigger and Grady, 1982). Furthermore, the auxiliary role of the feeding is in accordance with the study of van Loosdrecht and Heijnen (2002), which suggested that cells grown under long feeding periods retained the same kinetic characteristics as when grown under pulse feeding conditions.

The change from pulse feeding to continuous feeding significantly reduced the value of the half saturation coefficient for storage, $K_{STO,}$ while increasing the corresponding K_S value for the SBR operation at the same sludge age. Half saturation coefficients are now commonly evaluated as indicators of enzymatic activity, which is responsible for substrate transport and regarded as an indication of substrate affinity of microorganisms. In other words, the affinity increases with the higher activity of transport enzymes, corresponding to lower values for the half saturation coefficient, K_S (Daigger and Grady, 1982; Kovarova-Kovar and Egli, 1998; Ferenci 1999). Substrate affinity and therefore K_S also depends on the type of substrate and the substrate removal mechanisms sustained in the system –i.e. the metabolic state of the species responsible for substrate utilization. Consortia of rapidly growing bacteria show higher affinity than slow growing species based on the competition principle (Stumm-Zollinger and Harris, 1971). Although biomass adapts itself to the environmental conditions, the adapted population structure may affect the value of the affinity coefficient (Lavallee et al., 2005). For acetate utilization under continuous feeding which induces low substrate concentration-low growth rate conditions, substrate affinity is lowered – i.e. K_S value is increased to 25 mg/L, to divert substrate towards storage. Conversely, substrate affinity for storage was increased with much lower K_{STO} values compared to pulse feeding conditions. This observations are not compatible with the findings of Ferenci (1999), suggesting that high growth rate and low substrate concentration lead to a high affinity transport system, i.e. low K_S values; It gives clear indication that the mechanism and kinetics of substrate utilization may be quite different for simultaneous growth/storage as opposed to conditions evaluated solely as direct growth.

Another significant observation relates to the level of the maximum growth rate coefficient, μ_{H1} defined for acetate on the basis of model calibration. Based on the concept of direct growth, a narrow range of 5.0–6.5 1/day was defined for μ_{H1} for domestic sewage and different wastewaters; the result was justified with the fact the new modelling approach considers readily biodegradable COD, S_S, in the growth rate expression and the nature of S_S should not be expected to show significant variation among different wastewaters. Acetate is also a simple/readily biodegradable substrate and yet, a much lower range of 1.5-2.5 1/day was found to define the corresponding μ_{H1} in this study. First of all, this level is in agreement with 1.0-3.5

1/day range obtained in similar studies involving storage of acetate (Krishna and van Loosdrecht, 1999a; Koch et al., 2000; Sin et al., 2005). Beccari et al, (2002) even calculated a μ_{H1} value as low as 0.36 1/day in a similar SBR system operated with pulse feeding of acetate. Studies experiencing simultaneous growth and storage using different substrates such as glucose (Insel et al., 2007) or soluble starch (Karahan et al., 2006) also reported μ_{H1} values of 2.0-3.0 1/day. The result in this study, supported by similar findings in the literature give a clear indication that the rRNA level of cells, usually linked to the maximum growth rate, is sustained at a much lower level when the substrate is jointly utilized for storage along with direct microbial growth.

6.1.6 Interpretation of the modelling results

Model evaluation of experimental data justified the concept of variable kinetics in the utilization of acetate as a function of culture history (sludge age) and feeding regime. The rate coefficients for storage and direct growth (k_{STO}; μ_{H1}) were observed to vary with the selected sludge age, while the feeding regime mainly affected the affinity for acetate – i.e. the corresponding half saturation coefficients (K_{STO}; K_{S1}).

The maximum specific growth rate for acetate was evaluated as 1.5-2.5 1/day depending on the selected sludge age, much lower that the range of 5.0-6.5 1/day commonly associated with the utilization of readily biodegradable COD in domestic sewage and other compatible wastewaters. This observation indicates that the maximum growth rate is reduced when acetate is jointly utilized for storage along with direct microbial growth.

The impact of variable kinetics of biomass is of great importance in the selection of process and optimal operation of wastewater treatment plants by securing the effluent quality. From a practical standpoint, the results suggest that the conditions favouring the storage and/or growth could be triggered with the selection of continuous and/or batch processes under different sludge ages. The selection of optimal process would enhance biopolymer generation from sludge and/or production of energy via anaerobic digestion with downstream sludge treatment processes that could be associated with mixed liquor composition composed of active biomass and storage compounds.

6.2 Modelling of Kinetic and Stoichiometry for Slowly Biodegradable Substrate Removal in SBRs

6.2.1 Modelling of SolS removal

The mechanism described above is used to formulate the proposed model by Karahan et al. (2005) incorporating adsorption and the rate limiting hydrolysis processes in the case of starch given in Table 2.5 to proposed model for Readily Biodegradable COD given in Table 6.1. Thus, the model involves seven microbial processes with the related process kinetics and stoichiometry expressed in a way compatible with the previously adopted model as given in Table 6.4.

Table 6.4: Matrix representation of proposed model for Soluble Starch (S_S).

Component / Process	X_S	X_{ads}	S_S	X_{GLY}	X_H	S_O	Process rate
Adsorption	-1	1					$k_{ads}.X_S$
Hydrolysis		-1	1-f_{SI}				$k_H \frac{X_S/X_H}{K_X + X_S/X_H} X_H$
Growth on S_S	$-\frac{1}{Y_{H1}}$				1	$-\frac{1-Y_{H1}}{Y_{H1}}$	$\mu_{H1}.\frac{S_S}{K_{S1}+S_S}.X_H$
Storage of S_S			-1	Y_{STO}		$-(1-Y_{STO})$	$k_{STO}.\frac{S_S}{K_{S2}+S_S}.X_H$
Growth on X_{GLY}				$-\frac{1}{Y_{H2}}$	1	$-\frac{1-Y_{H2}}{Y_{H2}}$	$\mu_{H2}.\frac{K_{S1}}{K_{S1}+S_S}.\frac{X_{GLY}/X_H}{K_{STO}+X_{GLY}/X_H}X_H$
Endogenous respiration					-1	$(1-f_I)$	$b_H.X_H$
Endogenous Respiration of X_{GLY}				-1		-1	$b_{STO}.X_{GLY}$

The experimental data obtained from the starch fed SBRs (SBR 9-12) were used to reveal information on the biochemical transformations in the system. In this respect, the OUR profiles, together with the glycogen data were used to evaluate the stoichiometric relationships in the system. A typical OUR profile monitored during the reaction phase of SBR 9 fitted well with the selected modelling approach as given in Figure 6.7.

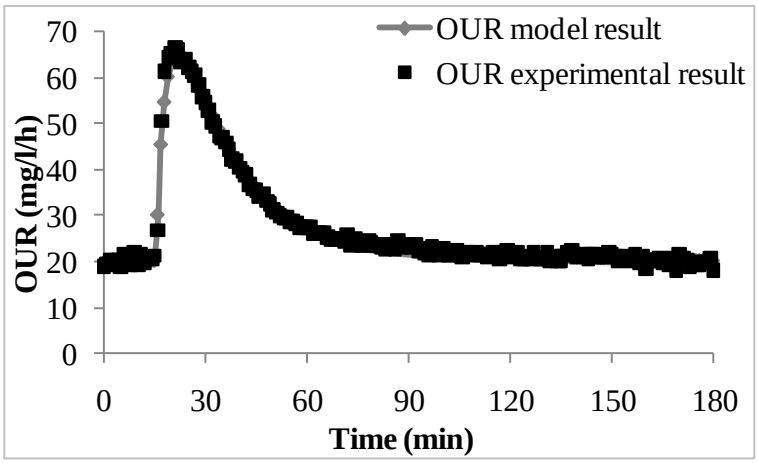


Figure 6.7: Model calibration of OUR profile for SBR 9.

The validity of the model was verified with the starch and glycogen data of the SBR 9. As reported by Karahan et al. (2006), it is not possible to differentiate the stored glycogen (X_{GLY}) and the starch adsorbed onto biomass (X_{Sads}) with the glycogen measurements. Therefore, the glycogen measurements account for the sum of the two sugars (X_{GLY} + X_{Sads}), especially just after the substrate addition. Accordingly, the model predictions for glycogen formation and consumption have also accounted for the amount of adsorbed starch in order to provide a better simulation of experimental results as given in Figure 6.8.

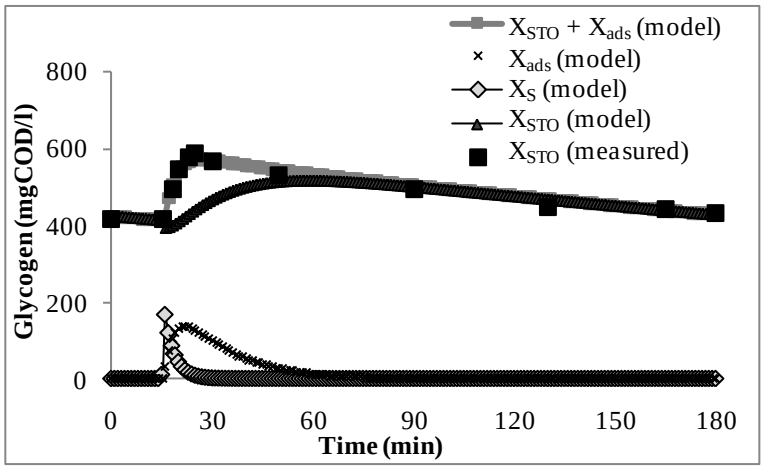
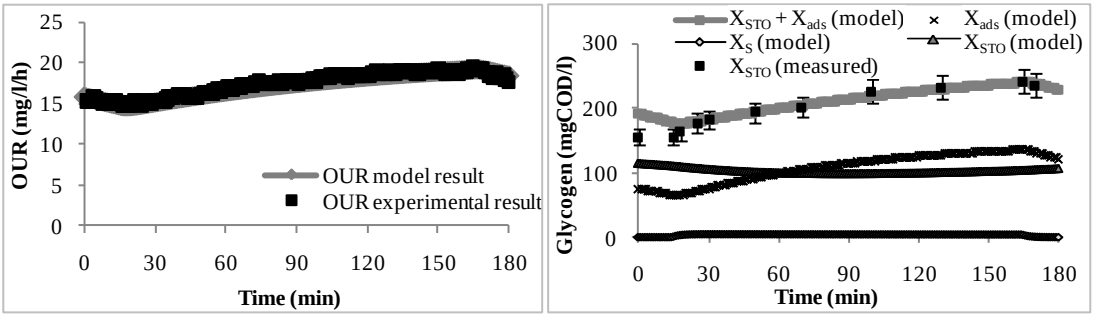


Figure 6.8: Model calibration of glycogen storage and utilization in SBR 9.

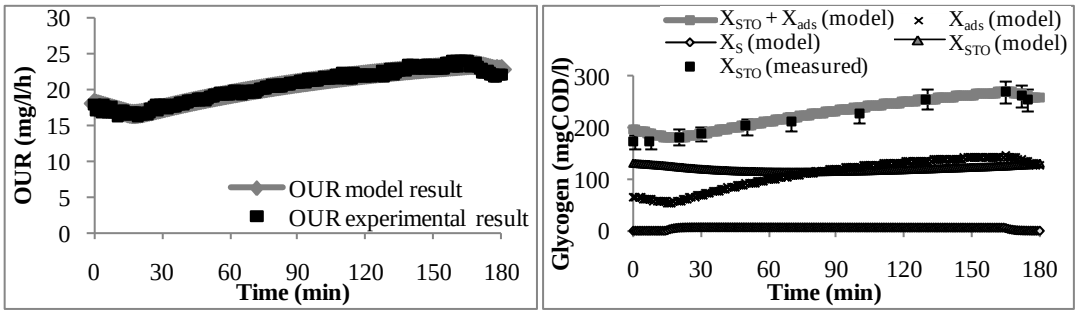
The model also presented good simulation results for OUR and glycogen profiles of continuously fed SBR (SBR 10) acclimated under same operating conditions as given in Figure 6.9, basically showing that the selected stoichiometric coefficients were appropriate also simulating the system in which the direct growth response was dominant mechanism.



(a) (b)

Figure 6.9: Calibration of (a) OUR and (b) glycogen profiles of SBR 10.

Although, the COD fraction used for storage was calculated as % 37 (Table 5.1) from the glycogen profile, the model simulation results showed that there is no storage under continuously feeding conditions as given in Figure 6.9b. In this experiment, the measured glycogen account only for the starch adsorbed onto biomass. This observation was verified with the model simulation results of SBR 12 which was also acclimated under continuously feeding conditions as given in Figure 6.10. The modelling results of continuously fed SBRs pointed out that the starch is not used for storage under continuously feeding conditions independently from the SRT.

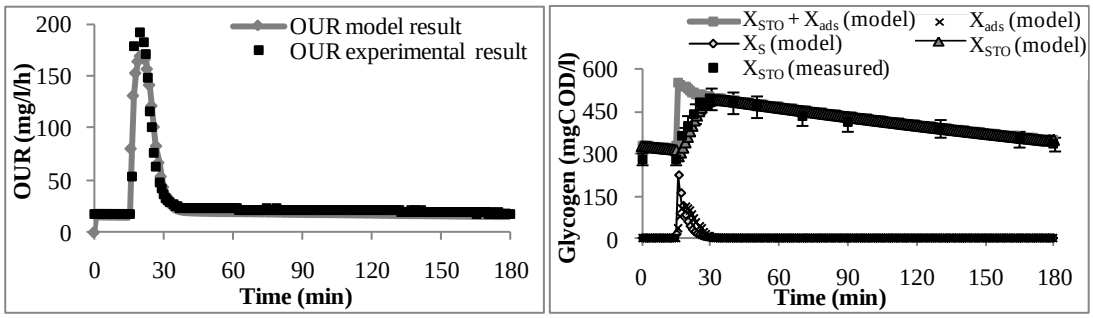


(a) (b)

Figure 6.10: Calibration of (a) OUR and (b) glycogen profiles of SBR 12.

6.2.2 Effect of the sludge age on the model structure

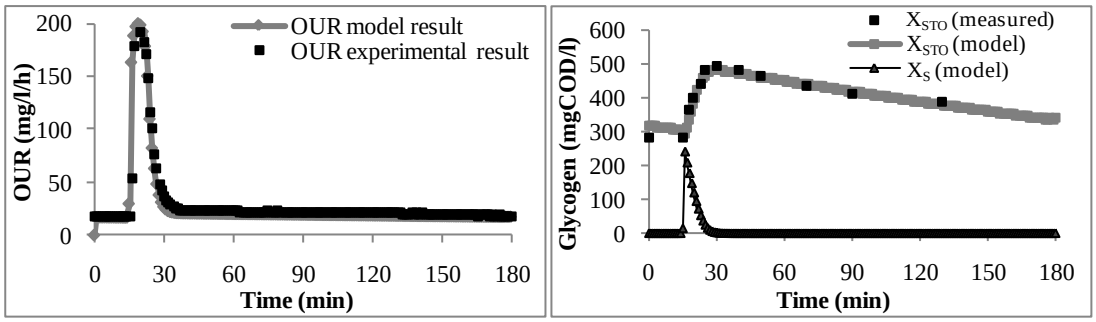
The glycogen measurements of SBR 11 did not give good simulation results with the yield values used for simulation of other SBRs fed with starch. Also the oxygen consumption for storage could not fit with experimental profiles as given in Figure 6.11.



(a) (b)

Figure 6.11: Model calibration of (a) OUR and (b) glycogen for SBR 11.

Since good simulation results were not obtained for the experimental results of SBR 11, the model was reconstructed with eliminating the adsorption process like proposed during the evaluation of the experimental results in Chapter 5. As seen from the simulation result of modified model given in Figure 6.12, the better simulation results were obtained for the SBR 11 with the elimination of the adsorption process. These model calibration results confirm the observation made from the glucose measurements of SBR 11 operated at the SRT of 2 days.



(a) (b)

Figure 6.12: Model calibration of (a) OUR and (b) glycogen profiles for SBR 11 without adsorption process.

6.2.3 Model calibration results

The stoichiometric and kinetic parameters providing best simulation results for SBRs fed with starch and the stoichiometric and kinetic parameters of previous experimental study performed with starch is given in Table 6.5.

As given in Table 6.5, the storage yield is compatible with the results of Karahan et al. (2006), although feeding pattern effect the extent of storage expressed with storage rate (k_{STO}) and the growth rates (μ_{H1} and μ_{H2}) depend on the SRT implemented to biomass.

Table 6.5: The stoichiometric and kinetic parameter obtained from model simulations.

Operating Parameter	**Units**	**Set 9**	**Set 10**	**Set 11**	**Set 12**	**Karahan et al., 2006**
SRT	1/day	8	8	2	2	5
Feeding Pattern	-	Pulse	Continuous	Pulse	Continuous	Pulse
COD loading	mgCOD/L.cycle	203	230	267	287	
Parameter						
k_{STO}	gSS/gX_H.day	25	5	25	5	25
K_{STO}	gX_{STO}/gX_H	1	0.1	1	0.3	0.40
K_{S1}	gCOD/L	20	20	20	20	20
K_{S2}	gCOD/L	2.0	10.0	2.0	4.0	2
k_{ads}	1/day	480	480	-	480	360
k_H	1/day	30	6	40	6	3
K_X	gCOD/gCOD	0.30	0.40	0.05	0.30	0.15
Y_{H1}	gCOD/gCOD	0.70	0.70	0.70	0.70	0.79
Y_{H2}	gCOD/gCOD	0.79	0.79	0.79	0.79	0.84
Y_{STO}	gCOD/gCOD	0.91	0.91	0.91	0.91	0.91
μ_{H1}	1/day	2.40	2.40	4.00	4.00	3.0
μ_{H2}	1/day	2.40	1.00	3.50	3.00	4.0
b_H	1/day	0.20	0.20	0.20	0.20	0.10
b_{STO}	1/day	0.05	0.05	0.05	0.05	0.05

7. INTERACTION BETWEEN THE REMOVALS OF DIFFERENT TYPE OF CARBON SOURCES UNDER DYNAMIC FEEDING

7.1 The Dynamic Behaviour of Activated Sludge Fed with Mixed Carbon Source

The dynamic profiles of the SBRs fed with mixture of starch and acetate, are given in Figure 7.1 in terms of COD, acetate and glucose removal in primary axis and the PHA and glycogen production in secondary axis.

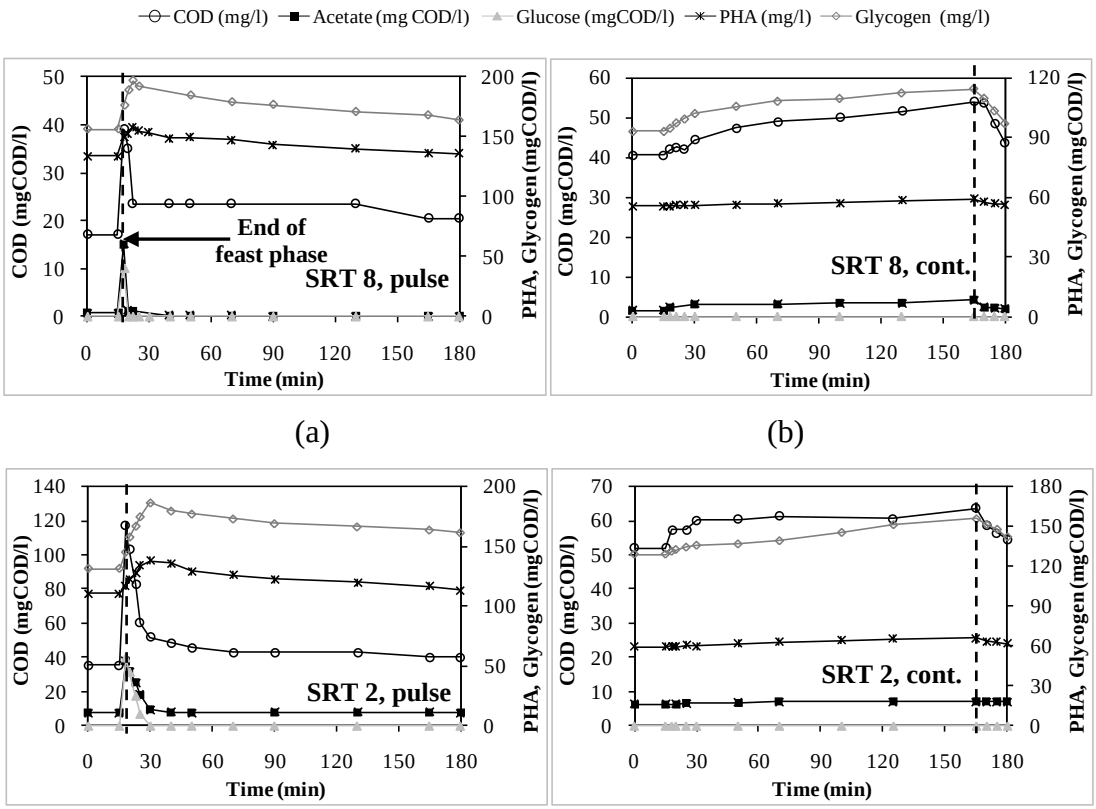


Figure 7.1: Dynamic behaviour of the activated sludge in (a) SBR 13, (b) SBR 14, (c) SBR 15 and SBR 16.

The experimental conditions and the performance of the SBRs are summarized in Table 7.1. As illustrated in Figure 7.1 and summarized in Table 7.1, the acetate and starch were removed simultaneously and quickly under pulse feeding; based on dynamic profiles in Figure 7.1, the feast phase lasted until the 7th minute and the 15th minute after the feeding at SRT 8 and 2, respectively (Set 13 and Set 15, respectively). Although, both carbon sources were removed quickly, the fraction of

the COD used for the storage was totally different. While the formed glycogen accounted for 80 – 83 % of the starch used, the PHA storage accounted for 43- 49 % of acetate used.

Table 7.1: Cycle Experiments: a) Experimental conditions and b) Observed behaviour.

a) Experimental conditions	**Set 13**	**Set 14**	**Set 15**	**Set 16**
Experimental SRT (day)	8.05	8.07	2.10	2.02
Biomass Concentration (mgVSS/L)	1190	1160	705	650
Initial Starch Concentration (mgCOD/L)	49	50	64	65
Initial acetate Concentration (mgCOD/L)	49	51	70	70
Initial Total COD Concentration (mgCOD/L)	98	101	134	135
F/M (gCOD/gVSS.d)	0.49	0.52	1.14	1.45
b) Observed Behaviour of SBRs				
Feast Phase (min)	7	150	15	150
Amount of Stored Glycogen (mgCOD/L.cycle)	41	21	56	27
Amount of Stored PHA (mgCOD/L.cycle)	24	4	28	7
COD fraction of Starch used for storage*	0.83	0.42	0.80	0.39
COD fraction of Acetate used for storage*	0.49	0.08	0.43	0.10
COD Removal (%)	90	78	85	80
Y_{OBS} (gCOD/gCOD)	0.35	0.34	0.59	0.56

*With the assumption of the storage of glycogen only from starch and PHA only from acetate

In the parallel SBRs operated with continuous feeding, the slow and gradually consumption of the carbon sources caused a slower formation of the storage polymers (Figure 7.1b and 7.1d). When considering the total COD concentration, the lower storage polymer production points out the presence of the direct growth as dominant mechanism in the continuously fed SBRs.

However, although under continuous feeding the rate of substrate removal was limited by the rate of substrate feeding, a significant amount of glycogen was still formed whereas the PHA storage was quite more decreased with respect to pulse feeding. Indeed, when comparing pulse vs. continuous feeding, the amount of glycogen (GLY) formed decreased by a factor of about 2 whereas the amount of PHA decreased by a factor of 4 or 6 at SRT 2 and 8, respectively. This observation is also supported with the average rates and yields calculated from the cycle experiments (Table 7.2).

Table 7.2: Rates and yields observed in cycle experiments.

Parameter	Set 13		Set 14		Set 15		Set 16	
	Starch	Acetate	Starch	Acetate	Starch	Acetate	Starch	Acetate
Specific Substrate Uptake Rate, $-q_s$ (mgCOD/gCOD.h)	250	251	11	11	280	256	28	26
Storage Polymer	GLY	PHA	GLY	PHA	GLY	PHA	GLY	PHA
Specific Storage Rate, q_p (mgCOD gCOD.h)	211	120	5	0.84	224	116	11	3
Specific Storage Polymer Consumption Rate, $-q_p$ (mgCOD/gCOD.h)	7	4	42	7	9	9	60	17
$q_p/-q_s$	0.84	0.48	0.42	0.08	0.80	0.45	0.41	0.12

In Table 7.3, The $q_P/-q_s$ and $q_G/-q_s$ ratios for the PHA/acetate and glycogen/ starch couples calculated from experiments carried out under similar operating conditions, either with single or mixed substrate were compared: The PHA storage from acetate was clearly decreased when acetate was fed together with starch, than when it was the only carbon source, either under pulse or continuous feeding. On the contrary, within pulse conditions, the similar storage capacity was observed for the couple glycogen/starch, independent from the presence of the acetate. Under continuous feeding, glycogen storage was even slightly increased in the presence of acetate compared to experiments with starch as the only substrate.

Table 7.3: Comparison of the observed storage capacities for single substrate and mixed substrate fed SBRs.

Feeding Pattern	SRT	Carbon Source	$q_G/-q_s$ (glycogen/ starch)	$q_P/-q_s$ (PHA/ acetate)
Pulse Feeding	8	Starch + Acetate	0.84	0.48
		Starch	0.83	-
		Acetate	-	0.68
	2	Starch + Acetate	0.80	0.45
		Starch	0.80	-
		Acetate	-	0.58
Continuous Feeding	8	Starch + Acetate	0.42	0.08
		Starch	0.36	
		Acetate	-	0.15
	2	Starch + Acetate	0.41	0.12
		Starch	0.33	-
		Acetate	-	0.37

7.2 Molecular Compositions of SBRs Fed with Mixed Substrate

The results of the FISH analyses carried out with the activated sludge samples taken from the SBRs at steady state conditions were summarized in Figure 7.2. Most of the bacteria present in SBRs were identified by phylum specific probes. The considerable amount of *Actinobacteria*, *Betaproteobacteria*, *Gammaproteobacteria, Alphaproteobacteria* and *Cytophaga–Flavobacteria–Bacteroidetes* group were observed in all SBRs although Firmicutes and Chloroflexi phyla were detected in a lower amount. As seen in Figure 7.2, most of the bacteria were observed as belonging to *Actinobacteria* except the SBR 16, in which the *Betaproteobacteria* and *Gammaproteobacteria* became dominant.

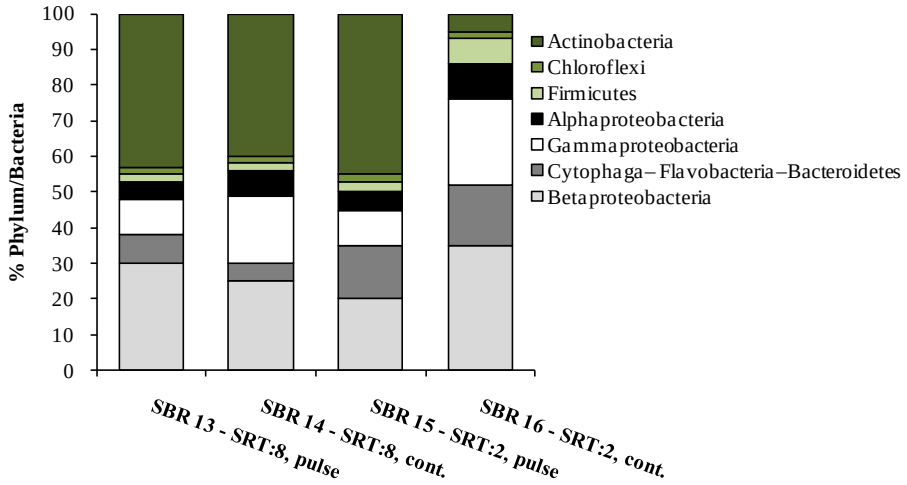


Figure 7.2: Microbial population dynamics in SBRs estimated by FISH analysis as a percentage of total bacteria in each SBR.

The phyla detected as abundant in SBRs were well matched with the results obtained from the experiments with single carbon source. In these studies, the *Betaproteobacteria, Alphaproteobacteria*, *Gammaproteobacteria*, and members of the *Cytophaga–Flavobacteria–Bacteroidetes* group were retrieved at the acetate fed SBRs, while the *Actinobacteria, Gammaproteobacteria*, *Betaproteobacteria* and *Alphaproteobacteria* were detected at starch fed SBRs. Thauera/Azoarcus group belonging to *Betaproteobacteria*, which was dominant and responsible for PHA storage in acetate fed SBRs at the SRT of 8 days, was detected in all SBRs. Nearly half of the Betaproteobacteria was originated from this group. In other words, this group was identified as at least 10% of all bacteria in each SBR. Although the same phyla were detected as dominant in mixed substrate and single substrate fed

environment, the abundance and type of the filamentous bacteria were very different at mixed substrate SBRs from the single substrate fed SBRs.

Type 021N and *Nostocoida limicola II* (*Alphaproteobacteria*) were found as dominant (nearly 50% of all bacteria) at the acetate fed SBRs, at the SRT of 2 days, while *Nostocoida limicola* belonging to *Actinobacteria* and a filamentous bacteria belonging to *Gammaproteobacteria* were detected in high abundance (nearly 30% of all bacteria) in the starch fed SBRs at the SRT of 8 days. Differently from these results, there was no Type 021N detected, while the *Nostocoida Limicola II* belonging to *Alphaproteobacteria* was only detected in Set 14. On the other hand, similar to starch fed SBRs, the abundance of the *Nostocoida limicola II* belonging to *Actinobacteria* and a filamentous bacterium belonging to *Gammaproteobacteria* were detected especially in continuously fed SBRs (Set 14 and Set 16) (Figure 7.3).

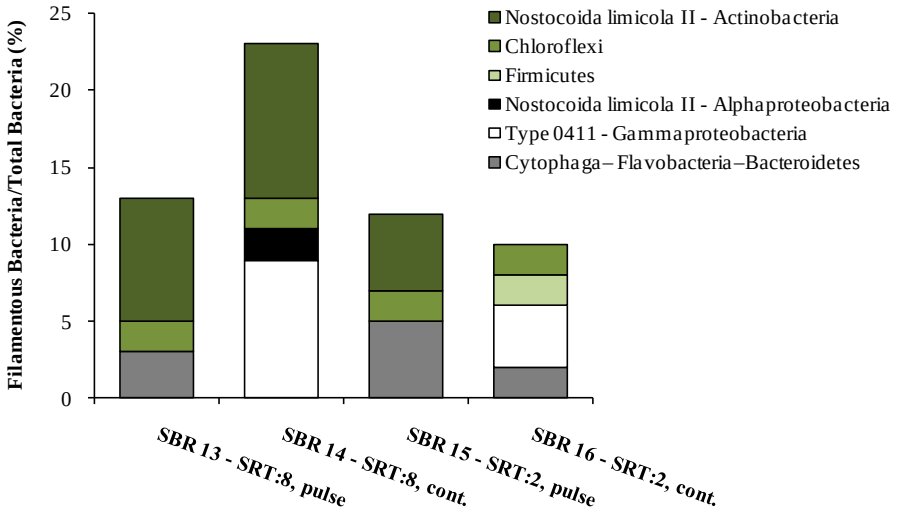
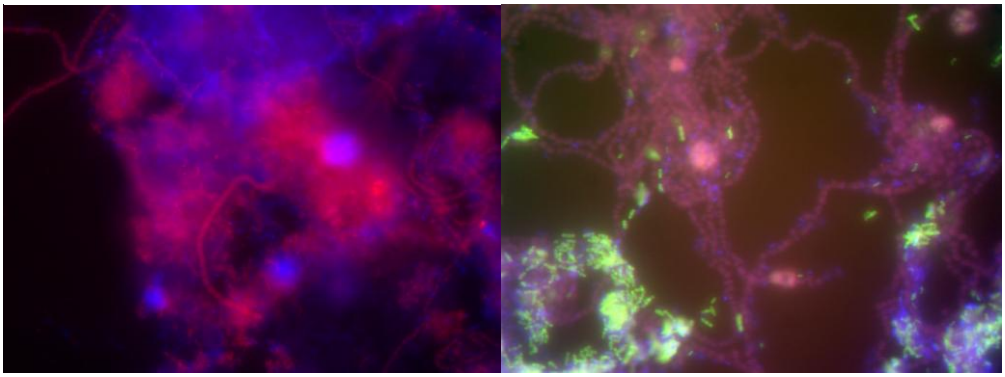


Figure 7.3: Amount of filamentous bacteria estimated by FISH analysis as a percentage of total bacteria in each SBR.

Also, the small amount of the Firmicutes and *Cytophaga–Flavobacteria–Bacteroidetes* were detected similar to the SBRs fed with starch as sole carbon source in addition to the Chloroflexi which was also detected in both single substrate fed SBRs. The representative FISH pictures of the most abundant filamentous bacteria were given in Figure 7.4.

Although high amount of the filamentous bacteria were detected in the continuously fed SBR at the SRT of 8 days, the observation of the well settling sludge (SVI<100 mL/g) has been supporting the observation of Martins et al. (2004). This author has

reported that sludge bulking independent from the feeding pattern or oxygen concentration during the experiments with starch was never experienced.



(a) (b)

Figure 7.4: Most abundant Filamentous Bacteria (a) *Nostocoida limicola* belonging to *Actinobacteria* (red) and (b) Type 0411 (red).

7.3 Effect of Dual Substrate Environment on the Formation of Intracellular Storage Biopolymers

The effect of dual substrate environment on the storage polymer formation was evaluated in SBRs operated under continuously feeding conditions at the sludge age of 8 days, a level commonly applied in activated sludge systems for different wastewaters. For this purpose, the SBR runs acclimated to different carbon sources were compared: one fed with acetate, the other with starch and the third one with the acetate/starch mixture. The common significant feature of all the three SBR runs was internal storage of biopolymers, regardless of the substrate type under continuous feeding. This observation confirmed that storage was not an exclusive outcome of pulse feeding, but occurred, although at a lower rate, in continuously fed systems. As expected, starch generated glycogen and acetate generated PHB, and both substrates developed a storage pool based upon the mass balance between the formation rate and internal utilization of the stored biopolymers. Furthermore, the pool exhibited a cyclic variation because a fraction of the substrate fed during each cycle was additionally stored followed by direct utilization for microbial growth. The operating parameters and performance of SBR experiments are outlined in Table 7.4.

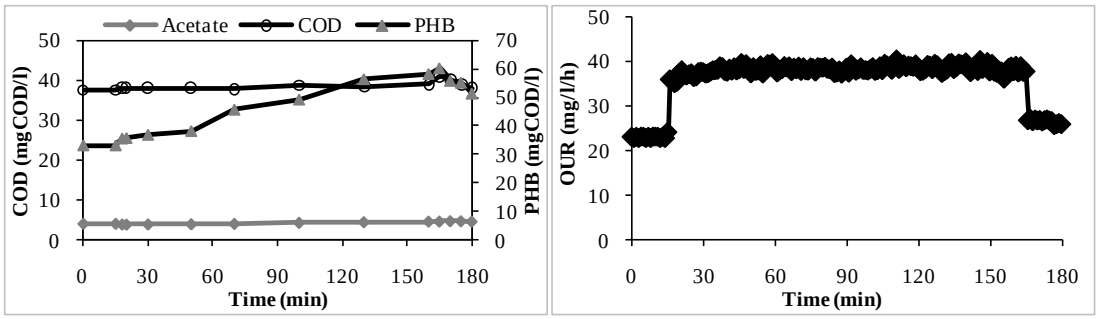
Table 7.4: The steady state characteristics of SBRs fed continuously at the sludge age of 8 days with different carbon sources.

	Run 1 (SBR 2)	**Run 2 (SBR 8)**	**Run 3 (SBR 14)**
a) Operating conditions			
Carbon source	Acetate	Starch	Mixed S.
Sludge age (days)	8.10	8.06	8.07
Initial starch concentration (mgCOD/L)	-	230	100
Initial acetate concentration (mgCOD/L)	180	-	102
b) Steady state parameters			
Average VSS (mgVSS/L)	1567	1790	2320
F/M (mgCOD/mgCOD.d)	0.49	0.54	0.37
Amount of stored glycogen (mgCOD/L.cycle)	-	86	42
Amount of stored PHB (mgCOD/L.cycle)	27	-	8
Glycogen/Starch ratio (mgCOD/mgCOD)	-	0.37	0.42
PHB/Acetate ratio (mg COD/mgCOD)	0.15	-	0.08
Acetate fraction converted to PHB (%)	18	-	9
Starch fraction converted to glycogen (%)	-	44	49

7.3.1 Utilization of acetate

Acetate is a simple, readily biodegradable organic compound, which is the common ingredient of domestic sewage and different industrial wastewaters. As in this study, it served as the selected substrate of many studies devoted to the understanding of storage mechanism (Dionisi et al., 2001) and enhanced biological phosphorus removal, where acetate, along with other volatile fatty acids, plays a vital role on process efficiency through formation and subsequent utilization of PHB. Typical concentration profiles of acetate, COD, PHB and OUR within a representative cycle of SBR operation at steady state are illustrated in Figure 7.5.

The first experimental observation is the significant difference between acetate and COD concentrations; acetate was almost completely removed while a COD level of around 35-40 mg/L persisted throughout the cycle, due to soluble microbial products (SMPs) generated as part of metabolic activities associated with substrate utilization and microbial growth (Orhon et al., 1999). This observation confirms again that the effluent COD should not be regarded as part of the unutilized influent biodegradable COD, but only residual COD, either initially present or generated within the biological reactor.



(a) (b)

Figure 7.5: Acetate utilization in SBR under continuous feeding (a) acetate, COD, PHB and glycogen profiles; (b) OUR profile.

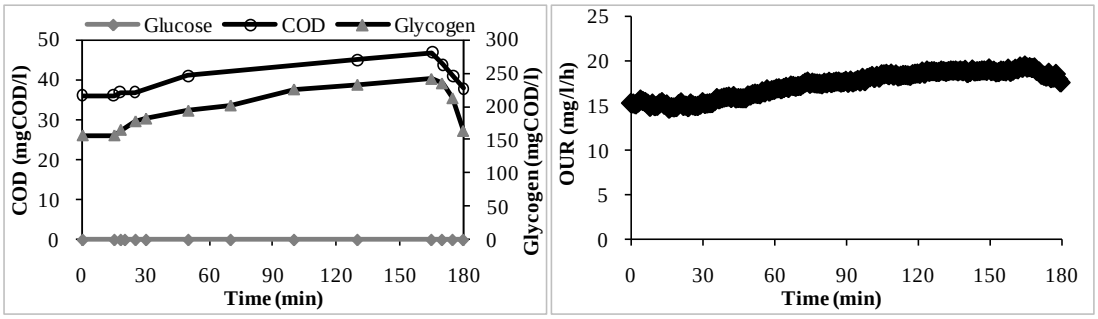
The results also indicated a PHB pool of around 30 mgCOD/L related to SBR operation under selected conditions. The pool gradually increased approximately to 60 mgCOD/L and it reverted back to the same level at the end of the cycle, indicating an average PHB generation of 27 mgCOD/L within each cycle. The resulting PHB/Acetate ratio – i.e. PHB COD generated compared to acetate COD fed – was calculated as 0.15. Adopting a storage yield, Y_{STO}, value of 0.85 mgCOD/mgCOD for acetate, derived from the modelling studies, around 18% of the acetate fed within each cycle was converted to PHB, while the remaining 82% was directly utilized for microbial growth.

7.3.2 Utilization of starch

Starch is a typical example of a slowly biodegradable substrate and it has been extensively used to investigate the mechanism of slow biodegradation (SanPedro et al., 1994). It requires hydrolysis by extracellular enzymes, converting it to simple readily biodegradable fractions that can be directly used for microbial growth. Recent studies have shown that it is rapidly removed from solution and adsorbed onto biomass before being hydrolyzed (Karahan et al., 2006).

The experimental results, illustrated in Figure 7.6, showed that its utilization mechanism was quite different in many ways from acetate, a simple substrate: (i) The SBR system sustained with starch feeding developed a much higher storage pool of around 150 mgCOD/L; glycogen was the storage biopolymer based on the nature of starch used as the sole organic carbon source. (ii) The average level of glycogen accumulation within each cycle was 82 mgCOD/L, which is much higher as compared with PHB storage associated with acetate. The stored glycogen was immediately used up for microbial metabolism/growth as soon as external starch

feeding stopped. (iii) Accordingly, a higher COD-based glycogen/starch ratio of 0.37 was observed, which suggested that around 44% of starch feeding was stored as glycogen under continuous feeding.



(a) (b)

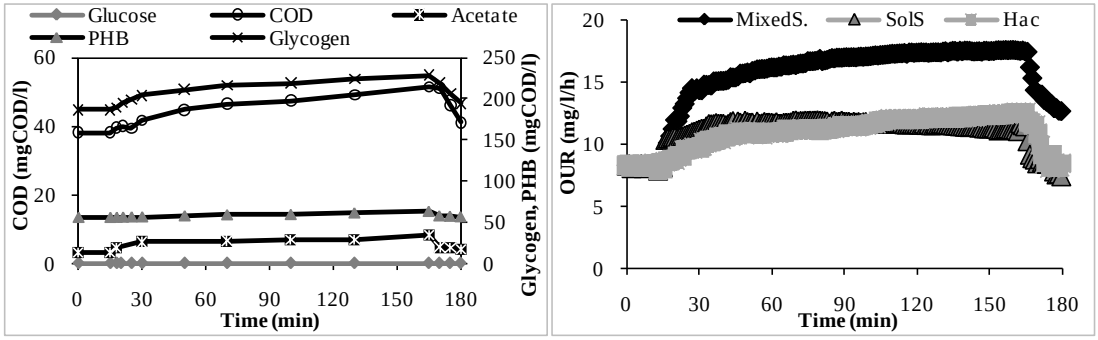
Figure 7.6: Starch utilization in SBR under continuous feeding (a) acetate, COD, PHB and glycogen profiles; (b) OUR profile.

The difference between the biodegradation characteristics for acetate and starch is well reflected on the corresponding OUR profiles: The one related to acetate indicates an almost constant level of OUR around 35 mgO_2/L.h, dictated by the utilization of available acetate – i.e. basically a single process. The gradually increasing OUR profile for starch includes both glycogen storage exhibiting a similar trend and the effect of hydrolysis limitation on substrate utilization.

7.3.3 Utilization of acetate/starch mixture

The COD level of the initial substrate feeding in the SBR experiment conducted with the acetate/starch mixture was adjusted to around 200 mgCOD/L by reducing the acetate and starch concentrations in single substrate runs. Resulting substrate and OUR profiles in a representative cycle are presented in Figure 7.7. Significant observations may be outlined as follows: (i) Substrate storage as PHB and glycogen persisted, presumably because the acclimated biomass sustained microbial fractions capable of performing metabolic functions associated with the formation of the two storage biopolymers. This observation was additionally supported by microbiological analyses conducted on the microbial communities in the SBR experiments fed by acetate, starch and the substrate mixture respectively. In fact, the dominant phyla of the SBR experiment fed with mixed substrate were identified as Actinobacteria and Betaproteobacteria: the first one was detected as the dominant phylum in starch-fed system and the second one, in acetate-fed system (Figure 7.8) (ii) Higher storage

pools were established with around 25% increase for glycogen and 60% increase for PHB, possibly due to slower utilization of the internal substrate pools for microbial growth. (iii) Considering the relative magnitude of both compounds in the substrate mixture, cyclic PHB accumulation was significantly reduced by almost 50% while glycogen accumulation exhibited a slight increase, suggesting that acetate was selectively directed towards direct utilization for microbial growth in the presence of starch, the slowly biodegradable component in the mixture.



(a) (b)

Figure 7.7: Utilization of acetate/starch mixture in SBR under continuous feeding (a) acetate, COD, PHB, glycogen and (b) OUR profile.

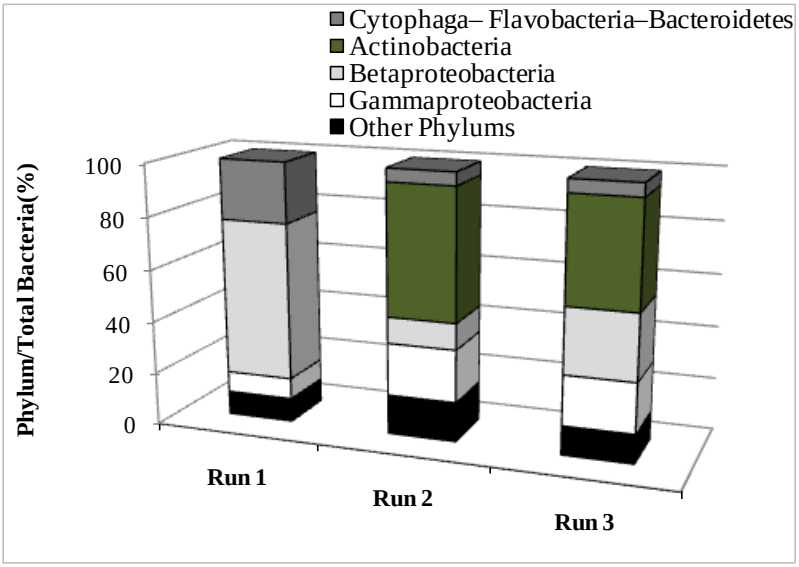


Figure 7.8: Bacterial Compositions of SBRs: (Run 1: starch; Run 2: acetate; Run 3: mixture).

7.4 Metabolic Characterization of the Removal Pathway of Different Substrates

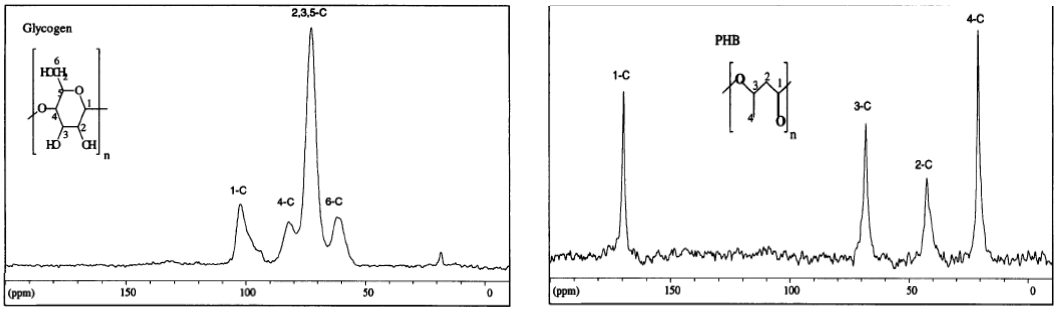
^{13}C NMR analysis

Nuclear Magnetic Resonance (NMR) has been used widely in studying the physical and chemical properties and the metabolism of PHA and glycogen in cells since it is

allowed to follow the fate of labelling from ^{13}C-enriched substrates through particular metabolic pathways (Maurer et al., 1997). The relevance of growth and storage as possible mechanisms for acetate and starch removal was investigated with individuation of the metabolic pathways involved in the formation of storage polymers as well as other pathways involved in acetate and starch removal depending on the carbon source by Carbon Nuclear Magnetic Resonance (^{13}C-NMR) analysis. The aim of this part of study was to investigate the aerobic metabolism of carbon sources in an activated sludge process, referring primarily to the role of storage compounds under dynamic conditions.

For this purpose, NMR experiments were performed with ^{13}C substrate on activated sludge samples acclimated to mixed carbon sources at SRT of 8 days. The ^{13}C-NMR analysis of starch and acetate was performed in the presence and absence of other substrate in order to establish the possible effect of the presence of the other substrate on the substrate removal mechanisms of ^{13}C labelled substrate.

The spectra gathered from the solid state NMR analysis were compared to well known ^{13}C resonances of solid state carbon NMR spectra of glycogen and PHB reported by Maurer et al. (1997) (Figure 7.9).



(a) (b)

Figure 7.9: Solid state NMR spectra of (a) pure natural glycogen and (b) synthetic PHB (Maurer et al., 1997).

The ^{13}C resonances of solid state acetate and starch are given in Figure 7.10 and Figure 7.11, respectively. The comparison among the obtained ^{13}C CP-MAS spectra showed in biomass fed with [1,2-$^{13}C_2$]acetate the presence of signals mainly relative to 4-C, 2-C, 3-C and 1-C of PHB at 19.47, 39.69, 66.27 and 169.1 ppm respectively. These signals were also present in biomass fed with ^{13}C acetate + unlabeled starch (red lines in figure), but with lower intensity than ^{13}C acetate sample.

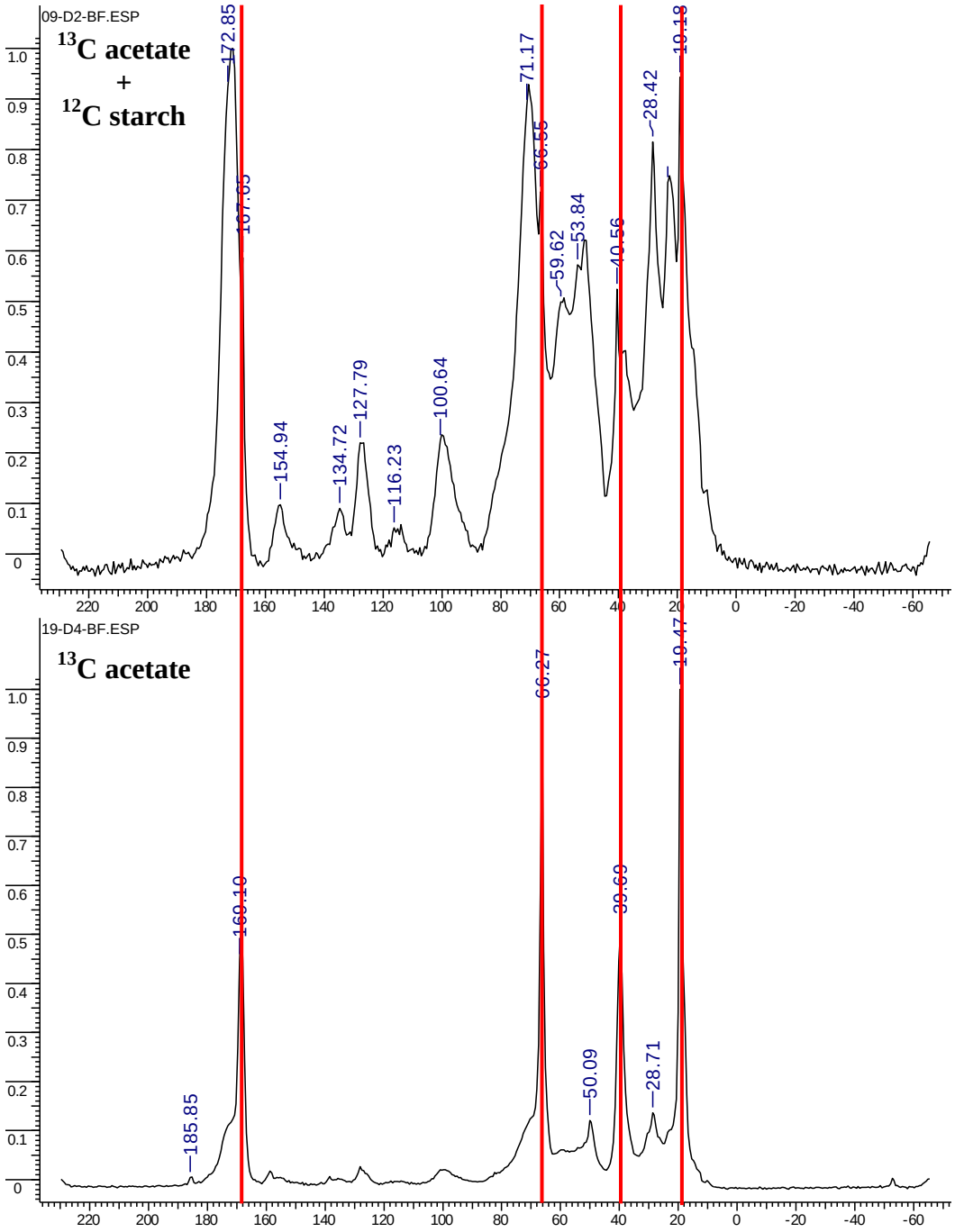


Figure 7.10: Solid state ^{13}C NMR spectra of biomass fed with [1,2-$^{13}C_2$] acetate (bottom) and [1,2-$^{13}C_2$] acetate + unlabeled starch (up) are shown. Red lines indicate the resonances of carbons of produced PHB.

The results seem to suggest an influence of starch feeding on ^{13}C acetate metabolism, whereas the feeding of biomass by acetate does not seem to affect the ^{13}C starch metabolism, in particular on the glycogen synthesis and protein synthesis, as showed in Figure 7.10 and 7.11.

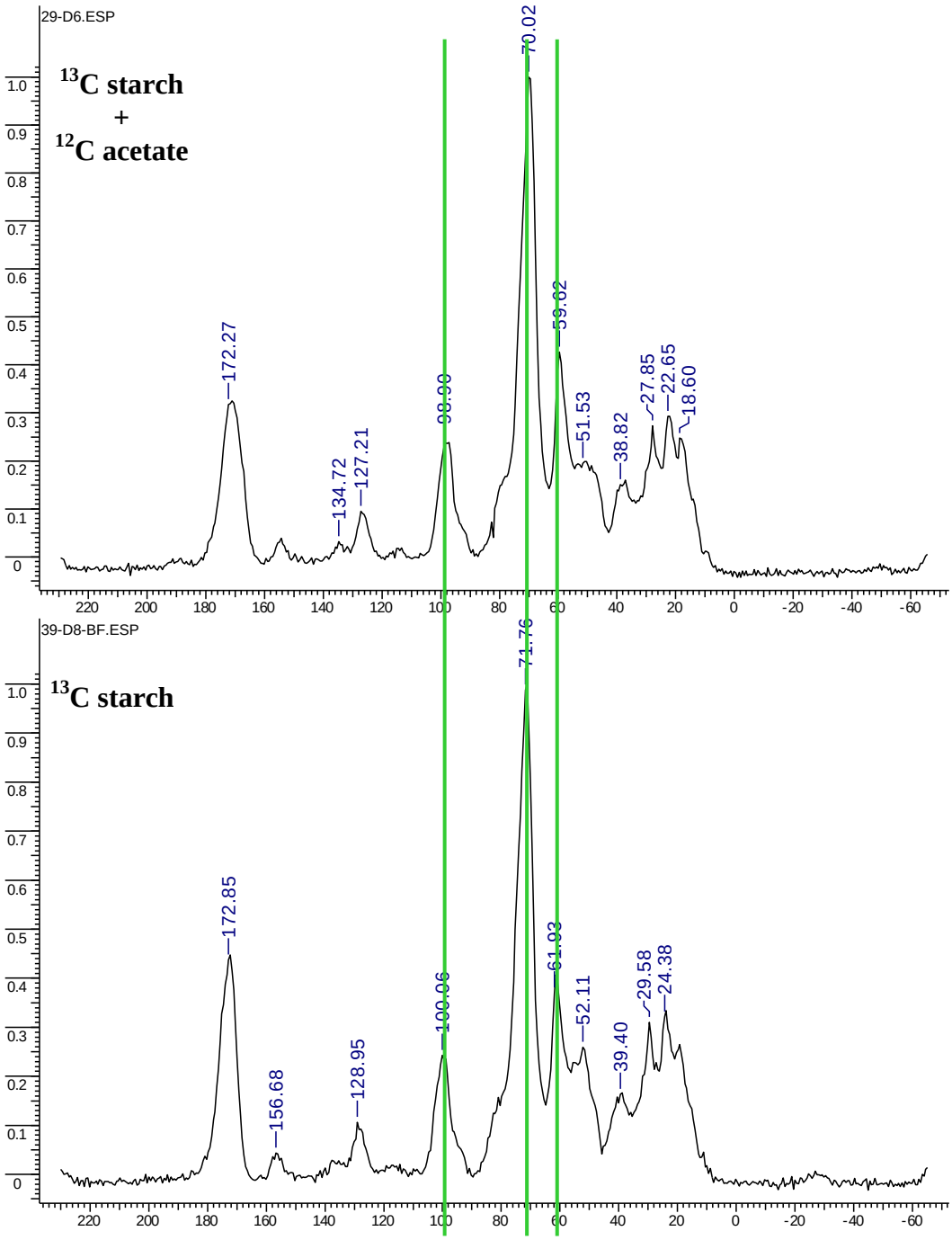


Figure 7.11: Solid state ^{13}C NMR spectra of biomass fed with [U-$^{13}C_2$] starch (bottom) and [U-$^{13}C_2$] starch + unlabeled acetate (up) are shown. Green lines indicate the resonances of carbons of produced glycogen.

In order to understand the influence of starch feeding on ^{13}C acetate metabolism, corresponding hydrosoluble extracts were analyzed with ^{13}C NMR high resolution spectroscopy. The ^{13}C NMR spectra of hydrosoluble extracts of biomass from SRT 8 fed with ^{13}C natural abundance acetate, ^{13}C acetate and ^{13}C starch are reported in Figure 7.12.

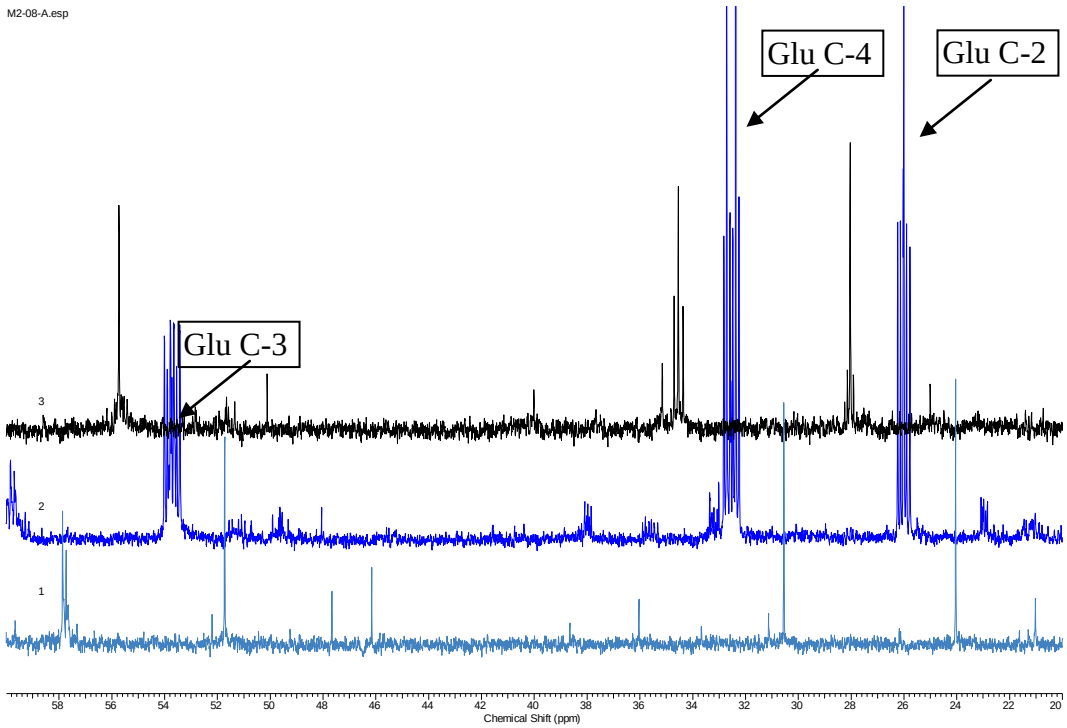


Figure 7.12: Typical ^{13}C NMR spectra of hydrosoluble extracts: (bottom) biomass fed with unlabeled acetate; (medium) biomass fed with ^{13}C acetate; (up) biomass fed with ^{13}C starch. The resonances at 24, 30.6 and 56.6 are relevant to C-2, C-4 and C-3 of glutamate respectively.

Two aminoacids, namely alanine (Ala) and glutamate (Glu), from hydrosoluble extracts were analyzed by ^{13}C NMR spectroscopy. The averaged ^{13}C enrichments are summarized in Table 7.5.

Table 7.5: The averaged ^{13}C enrichments of alanine (Ala) and glutamate (Glu).

Glu ^{13}C Isotopologues	STR =8 **^{13}C acetate**	STR =8 **^{13}C acetate + starch**	**Ala ^{13}C Isotopologues**	STR =8 **^{13}C acetate**	STR =8 **^{13}C acetate + starch**
0 = ^{12}C 1 = ^{13}C	%mol	%mol	0 = ^{12}C 1 = ^{13}C	%mol	%mol
00100	n.d	1	001	0.6	0.9
11100	3	6	100	0.8	0.8
11111	6	4	111	3.1	2.2
01111	1	3	011	0.6	1.0
11011	3	2	110	0.8	0.9
00011	6	8			
00111	2	4			
01100	n.d.	1			
Total ^{13}C Glu Isotopologues	21	29	**Total ^{13}C Ala Isotopologues**	5.9	5.8

Whereas the signals in ^{1}H-decoupled ^{13}C spectra of natural organic matter typically appear as singlets, many signals of the analyzed aminoacids appeared as multiplets, indicating the presence of multiply labelled isotopologue species with significant abundance. For example, the signal of C-2 of Glu was a pseudo triplet, where the central signal represents the [2-$^{13}C_1$]. The two flanking lines arose by ^{13}C-^{13}C coupling between the observed C-2 of Glu and a directly bound ^{13}C atom; the coupling constant of 34.5 Hz showed that the adjacent ^{13}C atom causing the signal-splitting is located at position 3. The relative signal intensities of the coupled signal pair indicated an abundance of mol % for [2,3-$^{13}C_2$]Glu (Table 7.5).

Small signals displaying a double doublet signature with coupling constants of 53.4 and 34.5 Hz were caused by Glu species carrying ^{13}C -label at positions 1, 2, and 3. The small doublet pair with a coupling constant of 53.4 Hz was due to the [1,2-$^{13}C_2$]-isotopologue. This analysis strategy was also applied for the signals of C4 and C3 of glutamate. The signals showing molar abundances below 0.5 mol % were not considered in the following analysis.

7.4.1 Analysis of alanine ^{13}C isotopologues

Alanine is the product of pyruvate transamination process. Consequently, alanine isotopologues reflect the labelling patterns of pyruvate derived from ^{13}C substrate metabolism. When the biomass is fed with $^{13}C_2$ acetate as the sole substrate, the microorganisms synthesize both acetyl-coA and the anaplerotic intermediates of the TCA to produce energy and biomass in aerobic condition.

The total amount of alanine isotopologues from $^{13}C_2$ acetate do not show substantial variations in respect to feast phase bioreactors fed with either $^{13}C_2$ acetate or not labelled starch. On the contrary, quantitative variations are showed by the analysis of single isotopologues (Table 7.5); in particular, a reduction of [1,2,3- $^{13}C_3$]Ala in the $^{13}C_2$ acetate + starch in respect to $^{13}C_2$ acetate fed biomass was showed. The production of [1,2,3- $^{13}C_3$]Ala from $^{13}C_2$acetate is possible solely from the decarboxylation of a fully labelled four carbons intermediate. The synthesis of a [1,2,3,4-$^{13}C_4$] intermediate can derive either through the condensation of two $^{13}C_2$ units in one malate molecule, or through the activity of malate synthase catalyzing the condensation of one $^{13}C_2$ Acetyl-CoA and one $^{13}C_2$ glyoxylate unit produced through isocitrate lyase activity, or through reactions producing malate or succinate

starting from [1,2,3,4-$^{13}C_4$] Acetoacetyl CoA, as described by Ensign (2006) and reported in Figure 7.13.

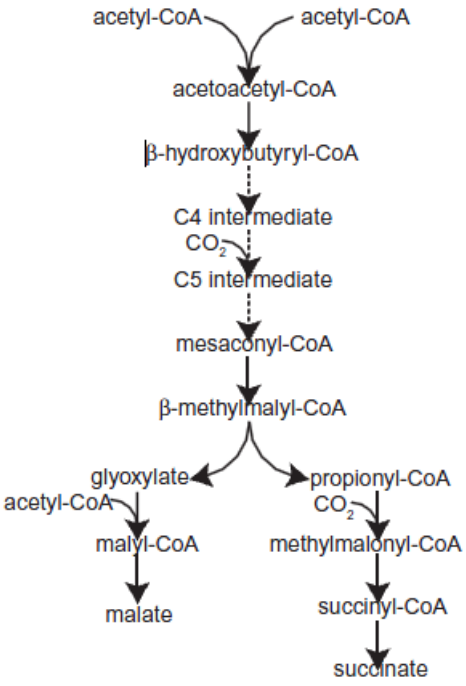


Figure 7.13: The synthesis of TCA cycle intermediate from central metabolite Acetyl CoA (Ensign, 2006).

In the famine phase, [1,2,3,4-$^{13}C_4$] acetoacetyl CoA can be produced from PHB depolymerization, in agreement with the model described by Salehizadeh and Van Loosdrecht (2004) as given in Figure 2.3.

Under aerobic dynamic feeding conditions, the growth of biomass and the storage of polymer occur simultaneously when there is an excess of external substrate. When all the external substrate is consumed, stored polymer can be used as carbon and energy source. However, in our experimental conditions, the amount of [1,2,3,4-$^{13}C_4$] acetoacetyl CoA derived from PHB depolymerization can be considered negligible as, at sampling time, PHB levels result still high although acetate levels are comparable with the before pulse ones.

The decrease of [1,2,3- $^{13}C_3$]Ala levels associated with a slight increase of other isotopologues in the $^{13}C_2$ acetate plus unlabeled starch in respect to the $^{13}C_2$ acetate fed biomass, suggests a glucose effect on the TCA reutilization of pyruvate, mainly derived from malate through malic enzyme activity, as an anaplerotic substrate, trough pyruvate carboxylase activity.

The results suggest that the starch derived glucose metabolism do not show a direct effect on the total alanine synthesis from $^{13}C_2$ acetate, as demonstrated by the unchanged isotopologues total amount, while inducing a diversification in the fluxes of the anaplerotic pathways with an increase of the pyruvate reutilization in the TCA.

To support this hypothesis, the results obtained in experiments utilizing [U-^{13}C] starch as substrate show the influence of acetate on alanine synthesis from [U-$^{13}C_6$] glucose. In fact, [1,2,3- $^{13}C_3$]Ala levels are decreased in the [U-^{13}C] starch + unlabeled acetate fed biomass in respect to the [U-^{13}C] starch fed one (1.3 %mol and 2 %mol, respectively). These results suggest that the biomass is conditioned to synthesize the anaplerotic substrates of TCA starting from acetate also in the presence of glucose.

7.4.2 Analysis of glutamate ^{13}C isotopologues

Glutamate is produced from 2-keto-glutarate through an oxidative transamination process. Then the glutamate isotopologues reflect the labelling pattern of the TCA produced 2-keto-glutarate. Utilizing [1,2-$^{13}C_2$] acetate as substrate, the TCA model involves the condensation of an oxaloacetate molecule with one [1,2-$^{13}C_2$] Acetyl-CoA producing [4,5-$^{13}C_2$]citrate. [4,5-$^{13}C_2$]2-keto-glutarate is then produced from isocitrate through the activity of isocitrate dehydrogenase. Starting from succynil-CoA a symmetric succinate molecule is formed, this latter characterized by a 50% scrambling of the labelling leading to the production of [1,2-$^{13}C_2$] or [3,4-$^{13}C_2$]succinate. The following sequential production of fumarate, malate and oxaloacetate will maintain the original succinate labelling pattern. Malate or oxaloacetate can be utilized in the cataplerosis processes to produce three carbon atoms intermediates, like pyruvate and phosphoenol pyruvate. Furthermore, 2-keto-glutarate, succinate, malate and oxaloacetate represent anaplerotic entry points of intermediates synthesized in different pathways, with labelling patterns reflecting the production pathways. Different glutamate isotopologues will then be produced from different TCA cycles, labelling scrambling and labelling patterns of anaplerotic intermediates.

The analysis of ^{13}C glutamate isotopologues (Table 7.5) of [1,2-$^{13}C_2$] acetate experiment shows that two isotopologues, like [1,2,3,4,5-$^{13}C_5$] and [4,5-$^{13}C_2$] glutamate, agree with a condensation of [1,2,3,4,- $^{13}C_4$]oxaloacetate or unlabeled

oxaloacetate with a [1,2-$^{13}C_2$] Acetyl-CoA, respectively. The levels of these isotopologues suggest same probability for citrate synthesis by [1,2,3,4,-$^{13}C_4$]oxaloacetate or unlabeled oxaloacetate. [1,2,3,4,5-$^{13}C_5$] glutamate production is in agreement with the presence of [1,2,3-$^{13}C_3$] alanine isotopologues and confirms the production of a TCA intermediate, [1,2,3,4,-$^{13}C_4$]malate, dependent on [1,2-$^{13}C_2$] glyoxylate and [1,2-$^{13}C_2$] Acetyl CoA condensation. The malate thus produced can cycle in the TCA and through the condensation between [1,2,3,4,-$^{13}C_4$]oxaloacetate or unlabeled oxaloacetate with [1,2-$^{13}C_2$] Acetyl-CoA, derived from [1,2-$^{13}C_2$] acetate, can either produce [1,2,3,4,5-$^{13}C_5$] and [4,5-$^{13}C_2$] glutamate or [1,2,3-$^{13}C_3$] or unlabeled pyruvate and, subsequently, [1,2,3-$^{13}C_3$] or unlabeled alanine.

The levels of [1,2,3,4,5-$^{13}C_5$] and [1,2,3-$^{13}C_3$] glutamate suggest that the probability of [1,2,3,4,-$^{13}C_4$]oxaloacetate and [1,2-$^{13}C_2$] Acetyl-CoA condensation is twice higher than that with unlabeled Acetyl-CoA.

7.4.3 Results of the ^{13}C NMR analysis

The comparison between two experiments performed with [1,2-$^{13}C_2$] acetate, as unique source of substrate, and [1,2-$^{13}C_2$] acetate + unlabeled starch shows:

- The formation of [4,5-$^{13}C_2$] glutamate, with [1,2-$^{13}C_2$] acetate + unlabeled starch as substrates, is higher when compared to the [1,2-$^{13}C_2$] acetate experiment;
- The [1,2,3,4,5-$^{13}C_5$] glutamate levels are lower than that observed in the [1,2-$^{13}C_2$] acetate experiment;
- The [1,2,3-$^{13}C_3$] glutamate levels are higher than that observed in the [1,2-$^{13}C_2$] acetate experiment;
- The ratio among the [1,2,3,4,5-$^{13}C_5$] and [1,2,3-$^{13}C_3$] glutamate levels, with [1,2-$^{13}C_2$] acetate + unlabeled starch as substrates, results to be lower as compared to the [1,2-$^{13}C_2$] acetate experiment;
- The [1,2,3,4-$^{13}C_4$] glutamate level, originated by re-cycling pyruvate through pyruvate carboxylase activity in the TCA cycle, is higher than that observed in the [1,2-$^{13}C_2$] acetate experiment. In Figure 7.14, the reconstruction of labelling pattern of [1,2,3,4-$^{13}C_4$] glutamate is reported.

- The total ^{13}C glutamate isotopologues measured in the [1,2-$^{13}C_2$] acetate + unlabeled starch experiment result to be higher than that measured in the [1,2-$^{13}C_2$] acetate experiment (29 % mol and 21 % mol, respectively).
- A constant flux of [1,2-$^{13}C_2$] Acetyl-CoA in the different isotopologues of glutamate is demonstrated by the sum of isotopologues of glutamate containing [4,5-$^{13}C_2$] (21 %mol versus 20 %mol in the [1,2-$^{13}C_2$] acetate experiment).
- An increase in re-cycling of [1,2,3-$^{13}C_3$] pyruvate, originated from [1,2,3,4,-$^{13}C_4$] malate through the malate synthase (anaplerosis), is demonstrated by the isotopologues of [2,3,4,5-$^{13}C_4$] glutamate (3 %mol versus 1 %mol in the [1,2-$^{13}C_2$] acetate experiment).
- An invariant TCA flux related with anaplerotic [1,2,3,4,- $^{13}C_4$] malate, is demonstrated by the sum of isotopologues of [1,2,3-$^{13}C_3$] and [1,2,3,4,5-$^{13}C_5$] glutamate (10 %mol versus 9 %mol in the [1,2-$^{13}C_2$] acetate experiment).

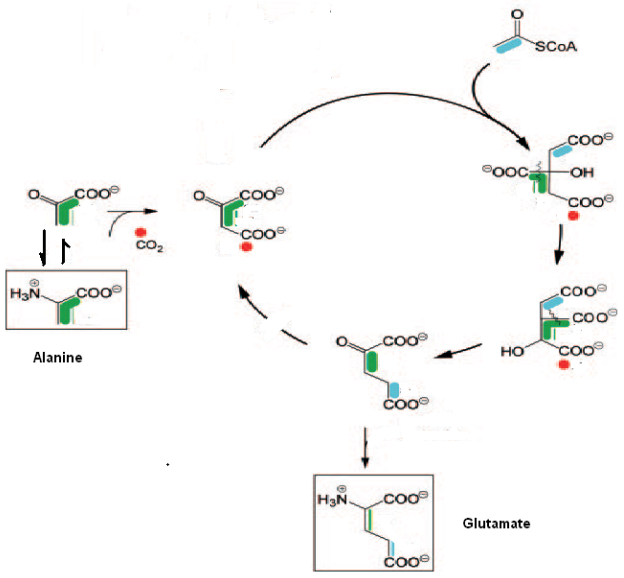


Figure 7.14: Reconstruction of the citric acid cycle from the experimentally determined labelling patterns of Ala and Glu (shown in boxes).

The labelling patterns of citric acid cycle intermediates were predicted on the basis of standard biosynthetic routes in bacteria. Bold bars connect ^{13}C-labeled atoms that were transferred from the same molecules of [1,2,3-$^{13}C_3$] pyruvate and [1,2-$^{13}C_2$] acetyl CoA derived from [1,2-$^{13}C_2$] acetate. The obtained results show that in conditioned biomass acetate is used as acetyl CoA for the synthesis of PHB for the

production of energy and biomass through the TCA cycle and for fulfilling the anaplerotic function by replenishing the intermediary metabolism. In particular, the results suggest that in acetate adapted biomass the replenishing of TCA intermediates, like malate, mainly occurs through the malate synthase activity, a member of the glyoxylate by-pass catalyzing the condensation of one molecule of glyoxylate with acetyl CoA. In this respect, a fundamental enzyme step results to be the isocitrate lyase activity that competes with isocitrate dehydrogenase for the substrate. The shift of utilization of isocitrate toward the formation of glyoxylate or 2-ketoglutarate seems to be regulated by the needs of the replenishment of anaplerotic intermediates.

7.4.4 Proposed central metabolic pathway for acetate metabolism

In a recent study, El –Mansi (2005) proposed a metabolic model that, on the one hand, implicates direct competition between phosphotransacetylase (PTA) and 2-ketoglutarate dehydrogenase (KGDH) for their common co-factor, free CoA (HS-CoA), and, on the other hand, between isocitrate dehydrogenase (ICDH) and isocitrate lyase (ICL) for their common substrate, isocitrate, as being essential for successful adaptation to biomass growth on acetate. In the model suggested by El-Mansi (2005), the HS-CoA availability represented a mechanism of regulation in the partition of carbon flux at the level of KGDH, as well as the isocitrate at the level of ICDH and ICL in facilitating the activity of glyoxylate by-pass during growth on acetate. Since 2-ketoglutarate dehydrogenase (KGDH) catalyzes the transformation of 2-ketoglutarate in succinyl-CoA, its activity might depend on HS-CoA/ Acetyl-CoA ratio. In this experimental model, the excess of acetate is mainly stored through the synthesis of acetyl CoA and acetoacetyl CoA in PHB. Consequently, the synthesis of PHB competes for HS-CoA with KGDH activity. A hypothesized bottleneck at the level of 2-ketoglutarate dehydrogenase could facilitate a high intracellular level of isocitrate, which in turn could ensure the flux through the anaplerotic sequence of glyoxylate bypass regenerating NADPH, a cofactor central for the uptake of ammonia through glutamate synthase and for the synthesis of PHB. The inferred metabolic model is summarized in Figure 7.15.

The proposed acetate metabolism model by result of this study is based on the model proposed by El-Mansi (2005) for the aerobic growth of Escherichia coli on acetate: (1) the HS-CoA / Acetyl-CoA ratio controls the flux through phosphotransacetylase

(2) and ketoglutarate dehydrogenase (4). Isocitrate lyase (5) and malate synthase (6), in conjunction with reactions of the citric acid cycle, allowed for the net synthesis of anaplerotic malate from two molecules of acetyl-CoA via a pathway named the glyoxylate cycle.

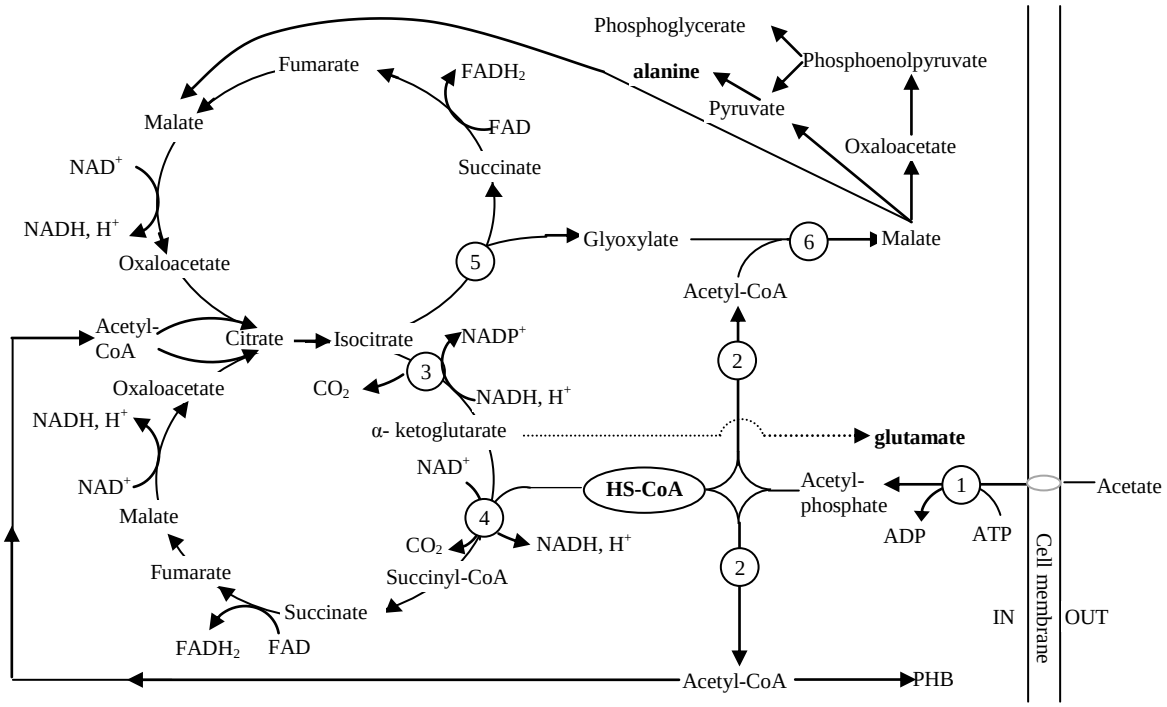


Figure 7.15: Representation of the proposed central metabolic pathways of intermediary and central metabolism for acetate metabolism (adapted from El-Mansi, 2005) (1): acetate kinase; (2): phosphotransacetylase; (3): isocitrate dehydrogenase; (4) 2-ketoglutarate dehydrogenase; (5): isocitrate lyase; (6): malate synthase.

When biomass was adapted to ^{13}C acetate + starch, our results showed a total increase in utilization of [1,2-$^{13}C_2$] Acetyl-CoA, produced by [1,2-$^{13}C_2$] acetate, in the TCA cycle and an anaplerotic pathway to produce energy and biomass, respectively. The reduction of PHB synthesis, observed in biomass adapted to ^{13}C acetate + starch, may be interpreted on the basis of the proposed hypothesis of a global control mechanism for the partition of free-CoA among various enzymes of central and intermediary metabolism involving, in particular, a competition for HS-CoA between KGDH activity and PHB synthesis pathway. An increase in the observed synthesis flux of glutamate from [1,2-$^{13}C_2$] acetate is in agreement with a reduced flux of Acetyl-CoA toward PHB synthesis, although a flux of Acetyl-CoA and pyruvate originated from glucose in TCA cycle was observed.

8. CONCLUSION AND RECOMMENDATIONS

Extensive research has been performed for the mechanistic description of biological processes occurring in activated sludge systems, in order to obtain best practices for their design and operation. This effort led to be recognized the storage phenomena as an important mechanism for the carbon source removal, particularly when the activated sludge experiences with highly dynamic conditions (aeration tanks with plug-flow configuration, selectors for bulking control, contact-stabilization processes, and sequencing batch reactors), so called the feast and famine conditions. Under dynamic conditions, cells are not able to instantaneously adapt their growth rate to the changing substrate concentration while, microorganisms can more quickly activate other mechanisms of substrate uptake like storage of substrate into specialized internal polymers, usually polyhydroxyalkanoate (PHA), especially from volatile fatty acids and glycogen from carbohydrates.

Although, the storage was suggested as the primary mechanism of substrate utilization in preliminary observations, the metabolic modelling studies showed that possibility of the simultaneously presence of storage and direct growth mechanisms under dynamic conditions depending on the operating conditions of activated sludge systems. In that sense, the mathematical description supplied by the model should regard the sludge history determined by operating conditions for investigating the fate of organic compounds both for microbial growth and storage depending on the relative magnitude of the corresponding stoichiometric and kinetic coefficients.

In this framework, the aim of this research was to get a deeper and better insight into the stoichiometry and kinetic of storage and growth processes. The investigation was designed to examining the effect of main operation conditions, namely sludge retention time, feeding pattern and carbon source, on microbial processes. The integrated application of chemical analyses and molecular technique (FISH: Fluorescent in Situ Hybridization) enabled to evaluate the effect of operating conditions on both metabolic processes and microbial composition.

Based on operational and performance data of SBRs fed with acetate under different operating conditions, it was concluded that the feeding pattern (pulse vs. continuous feeding) had a quite greater effect on the mechanism of substrate removal than on the microbial composition. Indeed, at any investigated OLR and SRT, acetate removal was quicker under pulse feeding than under continuous feeding and consequently the storage of polyhydroxyalkanoate (PHA) was the main mechanism of acetate removal under pulse feeding whereas it played a minor role under continuous feeding. On the contrary, pulse vs. continuous feeding did not cause a major change of dominant bacteria in the biomass, as determined by FISH. The combination of FISH with Nile Blue staining made it possible to confirm that the same dominant species were able to remove the acetate with or without intermediate PHA storage, depending on pulse or continuous feeding, respectively.

On the other hand, while maintaining a similar microbial composition for both types of feeding pattern at the same SRT and OLR, microbial composition significantly changed when SRT or OLR changed. Correspondingly at SRTs of 8 and 2 days, well-settling and bulking sludge were obtained, respectively. Because both well-settling and bulking sludge showed higher rate and yield of storage under pulse feeding than under continuous one, our results confirms that dynamic feeding and predominance of storage not necessarily select for floc-formers and well-settling sludge.

The experiment performed for the comparison of the short term and long term effects of acetate feeding on the microbial response showed that at steady state, storage was significant even under continuous feeding whereas pulse feeding brought a strong increase of both rate and yield of storage. Short-term and long-term effects were clearly distinguishable: the immediate adaptation of biomass coming from continuous feeding to a single spike accounted for a significant portion of the overall effect that was obtained after long acclimation to pulse feeding. The studies carried out with acetate was pointed out that a progressive increase of storage capacity is not necessarily due to a shift of microbial composition and should not interpreted by itself as an evidence of it. The presence or not of such an additional effect on microbial composition also depend on several other parameters, including substrate composition, organic loading rate and sludge age.

Acetate utilization induced PHB generation both under pulse and continuous feeding conditions. The results suggest that it would be misleading to evaluate the kinetics of acetate utilization without accounting for biopolymer storage in continuously fed activated sludge systems.

On the other hand, feeding regime was found as the decisive parameter to determine the utilization mechanism of starch: Although, it was almost totally stored as glycogen under pulse feeding, storage did not play a role in substrate removal under continuously feeding conditions as confirmed with modelling studies. Sludge age did not alter substrate utilization pattern although it affected the occurrence of adsorption process prior to the consumption of substrate for storage under pulse feeding conditions.

The SBR studies performed with mixture of acetate and starch indicated that, even with mixed substrates (acetic acid plus starch), pulse feeding strongly increased the storage of carbon sources compared to continuous feeding. However, the difference between continuous and pulse feeding was more important for PHA formation than for glycogen formation, because the PHA formation was increased by a factor of 4-6 (depending on SRT) whereas the glycogen formation was only increased by a factor of 2 (not dependent from SRT). From another point of view, more glycogen was always formed than PHB starting from the 50%-50% mixture of starch and acetate, which suggests that the storage yield of glycogen on glucose was higher than the one of PHA on acetate.

Both observations likely reflect the different metabolic pathways that are involved in the formation of glycogen and PHA, even though they could be also partially due to an overestimation of glycogen formation (because part of starch could be removed through adsorption onto the floc surface and so lumped with the glycogen after the their acid hydrolysis).

The observation of the simultaneous storage and consumption of PHA and glycogen under mixed substrate environment was supported by microbiological analyses, indicated that the acclimated biomass in the corresponding SBR system sustained microbial fractions capable of performing metabolic functions associated with the formation of the two storage biopolymers. PHB accumulation was reduced as acetate could be more readily used for direct microbial growth in the presence of starch.

The NMR studies confirmed that the amount of glycogen formed was unaffected from the presence or absence of acetate whereas storage of PHA was greatly decreased in the presence of starch. More in detail, when biomass was in contact with ^{13}C acetate + starch, NMR analysis showed an increase of utilization of [1,2-$^{13}C_2$] Acetyl-CoA towards TCA cycle and an anaplerotic pathway to produce energy and biomass, respectively. The corresponding reduction of PHB synthesis may be based on a global control mechanism for the partition of free-CoA among various enzymes of central and intermediary metabolism, involving the competition for HS-CoA between KGDH activity and PHB synthesis pathway. An increase in the observed synthesis flux of glutamate from [1,2-$^{13}C_2$] acetate is in agreement with a reduced flux of Acetyl-CoA toward PHB synthesis, although a flux of Acetyl-CoA and pyruvate originated from glucose in TCA cycle was also observed.

Consequently, the results obtained in this study underline a number of issues that are of practical significance for the operation of activated sludge systems. First of all, they showed that substrate storage is to be expected under continuous feeding conditions. This is quite important for the resulting rate of oxygen consumption because the oxygen demand of direct growth and storage exhibit different characteristics. It also affects the extent of available organic substrate during different phases of activated sludge operation, as utilization rates of different COD fractions and storage biopolymers are quite different. While this may not be so important for systems designed and operated for organic carbon removal, the type and extent of available substrate determines *the denitrification potential* (N_{DP}) and therefore directly affects the efficiency nitrogen removal.

The results of the study may also be evaluated for the operation of activated sludge systems to optimize the formation of storage biopolymers – glycogen in the case of starch removal. The collected experimental data clearly suggest system operation under pulse feeding, at sludge ages higher than eight days. Among different activated sludge process configurations, the sequencing batch reactor, as tested in the study, appears to offer the best operation flexibility for maximum generation of storage biopolymers.

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