THE DEVELOPMENT AND USE OF COMBINED CULTURES FOR THE TREATMENT OF LOW STRENGTH WASTEWATERS

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ABSTRACT

THE DEVELOPMENT AND USE OF COMBINED CULTURES FOR THE TREATMENT OF LOW STRENGTH WASTEWATERS

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This study was carried out to develop combined cultures which were composed of anaerobic and aerobic cultures, and could survive and operate under alternating aerobic and/or microaerobic / anaerobic conditions in semi-continuous and Upflow Sludge Blanket (USB) reactors. Granular combined cultures with median diameter of 1.28-1.86 mm and 0.8 mm were developed from suspended anaerobic and aerobic cultures in semi-continuous and USB reactors, respectively. Significant specific methanogenic activity (SMA, 14-42 mL CH_4/g VSS.hr) and specific oxygen uptake rate (SOUR, 6-47 mg DO/g VSS.hr) values of combined granules in semi-continuous reactors were comparable to those of anaerobic and aerobic granules. Similarly, combined granules in USB reactors exhibited noteworthy SMA and SOUR values of 11-77 mL CH_4/g VSS.hr and 10-75 mg DO/g VSS.hr, respectively. Combined granules developed in semi-continuous reactors were found to overcome the drawbacks of both anaerobic and aerobic granules such as the need for long start-up and low stability, respectively.

Combined cultures were also developed from anaerobic granular and suspended aerobic cultures in three USB reactors aerated at 10 mL air/min for 4 hours/day (R2), every other day (R3) and 24 hours/day (R4). The use of combined cultures was found to be advantageous compared to the anaerobic granules for the treatment of low strength wastewaters. During municipal wastewater treatment at influent 5-day biochemical oxygen demand (BOD_5) concentration of 53-118 mg/L (Hydraulic retention time, HRT: 0.75 day), combined cultures in R2, R3 and R4 exhibited average BOD_5 removal efficiencies of 52, 75 and 76%, respectively. Combined granules developed in USB reactor also displayed significant BOD_5 removal efficiencies (66-68%) during municipal wastewater application (HRT: 0.75 day).

Combined cultures/granules developed in USB reactors might be proposed as an alternative for municipal wastewater treatment due to their advantages such as achievement of required discharge standards, prevention of biomass loss / settleability problems unlike activated sludge systems and possible methanogenic activity as well as high settling characteristics comparable to those of anaerobic granules.

Keywords: Aerobic, Anaerobic, Microaerobic, Granulation, Municipal Wastewater

ÖZ

BİRLEŞİK KÜLTÜR GELİŞTİRİLMESİ VE DÜŞÜK KİRLİLİKTEKİ ATIKSULARIN ARITIMINDA KULLANILMASI

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Bu çalışma, yarı-sürekli ve yukarı akışlı çamur yataklı (YAÇY) reaktörlerde anaerobik ve aerobik kültürlerden oluşan ve değişken döngüsel aerobik ve/veya mikroaerobik / anaerobik koşullarda çalışabilen birleşik kültürler geliştirmek için yapılmıştır. Medyan boyu sırasıyla 1,28-1,86 mm ve 0,8 mm olan granüler birleşik kültürler yarı-sürekli ve YAÇY reaktörlerde askıda anaerobik ve aerobik kültürlerden geliştirilmiştir. Yarı-sürekli reaktörlerdeki birleşik granüllerin spesifik metanojenik aktivite (SMA, 14-42 mL CH_4/g UAKM.saat) ve spesifik oksijen tüketim hızı (SOTH, 6-47 mg DO/g UAKM.saat) değerleri sırasıyla anaerobik ve aerobik granüllerle karşılaştırılabilir düzeyde bulunmuştur. Benzer şekilde, YAÇY reaktörlerde geliştirilen birleşik granüller yüksek SMA (11-77 mL CH_4/g UAKM.saat) ve SOTH (10-75 mg DO/g UAKM.saat) aktiviteleri göstermişlerdir. Yarı-sürekli reaktörlerdeki birleşik granüllerin hem anaerobik hem de aerobik granüllerin, sırasıyla, uzun başlatma süresi gereksinimi ve düşük stabilite gibi dezavantajlarını giderdiği anlaşılmıştır.

Birleşik kültürler günde 4 saat (R2), gün aşırı (R3) ve 24 saat (R4) 10 mL hava/dakika ile havalandırılan üç YAÇY reaktöründe anaerobik granüler ve askıda aerobik kültürler kullanılarak da geliştirilmiştir. Birleşik kültürlerin düşük kirlilikteki atıksuların arıtımında kullanımının anaerobik granüllere kıyasla avantajlı olduğu anlaşılmıştır. Evsel atıksu arıtımı sırasında 53-118 mg/L'lik giriş 5-gün biyokimyasal oksijen ihtiyacı (BOD_5) derişiminde (Hidrolik bekletme süresi, HBS: 0,75 gün), R2, R3 and R4'deki birleşik kültürler sırasıyla %52, 75 ve 76'lık ortalama BOD_5 arıtım verimlilikleri göstermişlerdir. Evsel atıksu uygulaması sırasında YAÇY reaktöründe geliştirilen birleşik granüller de kayda değer BOD_5 arıtım performansları (66-68%) sergilemiştir (HBS: 0,75 gün).

YAÇY reaktörlerinde geliştirilen birleşik kültürler/granüller istenilen deşarj standartlarını sağlaması, aktif çamur sistemlerinden farklı olarak biyokütle kaybını ve çökelebilirlik problemlerini gidermesi, anaerobik granüllerle karşılaştırılabilir düzeyde yüksek çökelme özelliğinin yanı sıra olası metanojenik aktiviteleri gibi avantajları nedeniyle evsel atıksu arıtımı için bir alternatif olarak önerilebilir.

Anahtar Kelimeler: Aerobik, Anaerobik, Mikroaerobik, Granül Oluşumu, Evsel Atıksu

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ABBREVIATIONS

BM	: Basal medium
BOD	: Biochemical oxygen demand
BOD_5	: 5-day biochemical oxygen demand
COD	: Chemical oxygen demand
DO	: Dissolved oxygen
ECP	: Extracellular polymer
HAc	: Acetic acid
HRT	: Hydraulic retention time
IA	: Image analyses
MLSS	: Mixed liquor suspended solids
MLVSS	: Mixed liquor volatile suspended solids
OLR	: Organic loading rate
OUR	: Oxygen uptake rate
SBR	: Sequential batch reactor
SCOD	: Soluble chemical oxygen demand
SLR	: Sludge loading rate
SMA	: Specific methanogenic activity
SOLR	: Space organic loading rate
SOUR	: Specific oxygen uptake rate
SRT	: Solid retention time
SS	: Suspended solid
SVI	: Sludge volume index
UASB	: Upflow anaerobic sludge blanket
USB	: Upflow sludge blanket
VFA	: Volatile fatty acids
VSS	: Volatile suspended solid

CHAPTER 1

INTRODUCTION

Microbial ecosystems with aerobic and anaerobic zones are found in many natural environments, such as sediments, soils, stratified lakes and seas, biofilms and bacterial colonies. At the interface of aerobic and anaerobic zones, the concentration of dissolved oxygen (DO) is often very low but still sufficient for the growth of aerobes and not high enough for total inhibition of anaerobes (Gerritse et al., 1992). This fact has arisen a new study area where wastewater treatment can be accomplished under aerobic or microaerobic (DO < 1 mg/L, Yerushalmi et al., 2001) conditions in a single vessel containing both anaerobic and aerobic cultures.

The possible co-existence of anaerobic and aerobic cultures in the same reactor under aerobic or microaerobic conditions might be attributed to the: 1) intrinsic tolerance of anaerobic cultures (Gerritse et al., 1990; Zitomer and Shrout, 1998), or 2) to the formation of anaerobic zones (shielding effect) (Wu et al., 1987; Field et al., 1995). Anaerobic environments can be expected to occur in the systems or inside the particulate matrices located even in well-aerated systems when there is an excess of readily metabolizable substrate compared to the oxygen supply; and when oxygen supply to deep layers is limited to the slow process of diffusion due to mixing barriers caused by biofilms, settled layers of particles or soil aggregates (Field et al., 1995). Therefore, formation of anaerobic zones, which might be disadvantageous for the aerobic treatment systems, turns out to be an advantage in the treatment systems where both anaerobic and aerobic metabolism is required.

The co-existence of anaerobes and aerobes was initially studied via the co-immobilization of the cultures in natural polymers such as calcium alginate or gel beads (Beunink and Rehm, 1988, 1990; Kurosawa and Tanaka, 1990; Gardin et al., 2001) or vermiculate support in nylon netting (Gerritse and Gottschal, 1992). Some investigators demonstrated the co-existence of anaerobic and aerobic cultures in packed-bed columns (Yerushalmi et al., 1999), in chemostats (Gerritse et al., 1990) and batch reactors (Zitomer and Shrout, 1998). In all these reactor studies, with either free or co-immobilized cultures of anaerobes and aerobes, it is observed that DO concentrations display alternating values. Due to the changing oxygen gradient alternating from aerobic and/or microaerobic to anaerobic conditions are achieved either through the reactor content or from bulk liquid to the depths of the immobilized co-cultures. Alternating aerobic / anaerobic conditions, therefore, support the co-existence and operation of anaerobic and aerobic cultures in the same reactor via developing supportive living conditions for each culture type.

Studies with co-immobilized cultures of anaerobes and aerobes reveal that the aerobic cultures grow in the oxygen-sufficient surface area, while the anaerobic ones grow in the oxygen-deficit central part of the gel beads (Kurosawa and Tanaka, 1990). Therefore, development of granules from anaerobic and aerobic cultures might be possible due to the alternating aerobic to anaerobic conditions achieved through the depths of the granules. In other words, granulation of anaerobic and aerobic cultures (without any support media) might be experienced in the same reactor as survival mechanisms of cultures.

Granulation process and its triggering mechanisms have been major study areas for more than 25 years (Hulshoff Pol et al., 2004). Its advantages (Speece, 1996) such as regular, dense and strong microbial structure, good settling ability, high biomass retention and tolerance to high organic loading rate / toxic shocks make granulation process popular in the wastewater treatment technology. There have been successful applications of granulation with either anaerobic or aerobic

cultures under anaerobic or aerobic conditions, respectively. However, on the other hand, both anaerobic and aerobic granulation technologies have some drawbacks. Anaerobic granulation needs long start-up, a relatively high operation temperature and is unsuitable for removal of nutrients (Lettinga et al., 1980; Liu et al., 2003a; Liu and Tay, 2004). Compared to anaerobic granules, aerobic granules have relatively low stability because of their fast-growth rate (Liu and Tay, 2004). Granulation of anaerobic and aerobic cultures (in the same reactor) appears to be promising for effective use of anaerobic zones by location of anaerobic cultures in the inner oxygen-free parts. Besides, granules developed from anaerobic and aerobic cultures might overcome the drawbacks of both anaerobic and aerobic granules. However, granulation from a mixture of anaerobic and aerobic cultures in the same reactor has not been studied so far except by Ferguson (1999) who investigated the perchloroethylene and benzene degradation but observed pelletization.

The advantages of using combined anaerobic and aerobic cultures in one reactor (i.e. coupled reactor) might be remarkable in treatment of several contaminants requiring sequential anaerobic and aerobic or anoxic mechanisms. Total mineralization of some polycyclic aromatic hydrocarbons and highly chlorinated solvents that require sequentially-operated anaerobic and aerobic or anoxic reactors were achieved in one coupled reactor by combined anaerobic and aerobic cultures (Beunink and Rehm, 1988, 1990; Gerritse and Gottshal, 1992). In addition, when considering in-situ technologies for soil/groundwater remediation where the oxygen-limited conditions prevail, it may be unfeasible to divide the system into two locales containing different cultures, which would not be the case for coupled reactors.

This new study area with combined anaerobic and aerobic cultures in one reactor also appears to be promising for improving the efficiency and reducing the costs compared to the conventional anaerobic or aerobic treatment systems. Coupled reactors may achieve lower effluent biochemical oxygen demand (BOD) values

than conventional anaerobic treatment processes and recover from organic shock loads more quickly (Zitomer and Shrout, 1998). Furthermore, coupled reactors (combined anaerobic and aerobic cultures) are potentially more energy efficient than conventional aerobic systems, requiring less energy for blower operation and producing significantly less biosolids to be handled, transported and disposed. Activated sludge system surely is the most widely used biological process for the treatment of municipal wastewaters (Rittmann and McCarty, 2001). However, the combined anaerobic and aerobic cultures might have the possible advantages of each culture type and treatment of municipal wastewaters with these cultures might be advantageous in terms of less aeration, lower biosolids and additional methane production. As a result, it might be worthwhile to investigate the treatment of low strength wastewater with combined anaerobic and aerobic cultures due to the possible advantages and ability to overcome the drawbacks of conventional activated sludge systems.

Studies with combined anaerobic and aerobic cultures cultivated in one reactor have been carried out since the last twenty years and literature information is limited to a few studies (Beunink and Rehm, 1988, 1990; Gerritse et al., 1990; Kurosawa and Tanaka, 1990; Gerritse and Gottschal, 1992; Zitomer and Shrout, 1998; Yerushalmi et al., 1999; Gardin et al., 2001). Besides, little is known about the possible applications of combined anaerobic and aerobic cultures, as well as the involved mechanisms.

The objective of this thesis is to develop combined anaerobic and aerobic cultures co-existing and operating under alternating aerobic / anaerobic (and in turn their transient microaerobic) conditions. It is hypothesized in this thesis that a starting mixture of anaerobic and aerobic cultures exposed to alternating aerobic and/or microaerobic / anaerobic conditions in the same reactor could survive and operate. Besides, these cultures would have the advantages of both anaerobic and aerobic treatment and would minimize the disadvantages of using anaerobic or aerobic cultures alone. The term ‘combined cultures’ is, therefore, defined in the thesis as

the mixture of anaerobic and aerobic cultures that could survive and operate under alternating aerobic and/or microaerobic / anaerobic conditions in the same reactor. Anaerobic conditions used herein also refer to the conditions within the granules as well as in the reactor content.

The scope of the thesis is given below:

1) To investigate the granulation of a mixture of suspended anaerobic and aerobic cultures under alternating aerobic and/or microaerobic / anaerobic conditions in semi-continuous and upflow sludge blanket (USB) reactors. In other words, to develop combined cultures of anaerobes and aerobes in granular form.

In this part of the study, the possibility of granulation under alternating aerobic and/or microaerobic / anaerobic conditions with a mixture of suspended anaerobic and aerobic cultures was investigated via semi-continuous and USB reactor studies. The optimum aeration protocol or oxygen doses leading to granulation were investigated. The optimum parameters such as substrate type and operational mode leading to granules of maximum sizes were examined. This part also covered the determination of the physical (settling velocity, particle size) and microbial (specific methanogenic activity, SMA and specific oxygen uptake rate, SOUR) characteristics of the granules developed under optimum conditions.

2) To investigate the treatment of low strength wastewaters in USB reactors with the combined anaerobic and aerobic cultures developed under alternating aerobic and/or microaerobic / anaerobic conditions. To determine the optimum / feasible aeration protocol for the treatment of original municipal wastewater with the developed cultures under mentioned conditions.

In this part of the study, the objective was to investigate whether the reactors composed of combined cultures of anaerobes and aerobes could be an alternative for the treatment of municipal wastewaters. This part covered the determination of

the aeration periods leading to the alternating aerobic and/or microaerobic / anaerobic conditions in the USB reactors and the development of combined anaerobic and aerobic cultures under mentioned conditions. The physical and microbial characteristics of the combined cultures developed in the USB reactors were also investigated.

CHAPTER 2

LITERATURE REVIEW

The literature review which is relevant to the scope of this thesis was reviewed and presented in this chapter.

2.1. The Co-existence of Anaerobic and Aerobic Cultures and its Applications in Environmental Engineering

Evidence in literature indicates that anaerobic and aerobic cultures can occur side by side in one reactor. Strict anaerobes can occur in anaerobic microniches located inside biofilms and aggregates or many have the ability to survive periodic exposure to oxygen (Field et al., 1995). The possibility of combined living or co-existence of anaerobic and aerobic cultures side by side, the reasons and the possible applications of these co-culture systems in environmental engineering are discussed in the following sections.

2.1.1. Oxygen Tolerance of Anaerobic Cultures

Oxygen is considered as a potential toxic compound to anaerobic treatment, especially for the acetogens and principally the methanogens that are usually regarded as strict anaerobes. However, some previous studies have demonstrated that some anaerobic bacteria have an oxygen tolerance and differ in their ability to withstand exposure to oxygen (Huser et al., 1982; Kato et al., 1993; Field et al., 1995).

2.1.1.1. Formation of Anaerobic Microniches or Shielding Effect

Survival of anaerobic cultures in oxygen-limited mixed cultures is often attributed to formation of anaerobic microniches in otherwise oxic environments. Oxygen has a low solubility and a slow diffusibility in water (Field et al., 1995). Consequently, when there is an excess of readily metabolizable substrate compared to the oxygen supply, anaerobic environments are created. Even in well-aerated systems, as soon as there is a barrier to mixing, the O_2 supply will become rate limiting. In wastewater treatment systems or during bioremediation of soils and sediments, such barriers are known to be caused by biofilms, settled layers of particles or soil aggregates, which provide resistance to convective mass transport. Thus, oxygen supply to deep positions is almost exclusively limited to the slow process of diffusion. Based on these observations, anaerobic microniches can be expected to occur in the systems or inside the particulate matrices located in oxic environments (Field et al., 1995). The presence of methanogens in aerated activated sludge suggests the existence of anaerobic microniches even in highly aerated environments (Huser et al., 1982; Lens et al., 1995). The cultivation of anaerobic granular sludge in an upflow anaerobic sludge blanket (UASB) reactor using aerobic activated sludge as seed has also been demonstrated (Wu et al., 1987) (Figure 2.1). The success can be attributed to presence of methanogens in the aerobic sludge. *Methanobacterium*, *Methanococcus* and *Methanosarcina* were observed in both the original aerobic activated sludge flocs and the anaerobic granular sludge formed. According to the fluorescent microscopic examination, several anaerobic nuclei were identified deep inside flocs of the original aerobic sludge (Figure 2.1). The anaerobic bacterium *Bacterioides* sp. was also detected at a depth of 800-900 from the aerobic granule surface (Tay et al., 2002a).

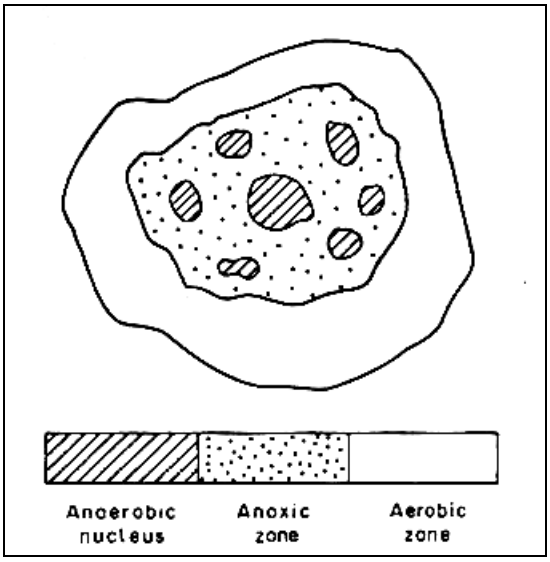


Figure 2.1. Illustration of an activated sludge floc having anaerobic nuclei (Wu et al., 1987).

The existence of anoxic zones in the deep inside of flocs or immobilized biofilms is due to the biofilms itself. Under steady-state conditions, zones of decreasing oxygen concentration will be formed towards the center of the biofilms. The depth of oxygen penetration will depend on the diffusion and consumption of oxygen. The diffusion will be most influenced by the oxygen concentration in the bulk liquid phase and the thickness of the biofilms, while the oxygen consumption is influenced by the presence of readily metabolizable substrates. The existence of oxygen-free microenvironments within biofilms has also been shown by utilizing microsensors. In experiments with carrageenan gel particles containing immobilized *Eschericchia coli* B, the oxygen penetration depth was 100 μm with glucose as substrate (Hooijmans, 1990; cited in Kato et al., 1993). Similarly, under bulk DO of 7 mg/L, oxygen concentration through gel beads decreased to zero at a depth of 300 μm (Kurosawa and Tanaka, 1990). Microprofiles have also been measured in nitrifying bacterial aggregates, where oxygen diffusion was

observed up to 100-300 μm (De Beer et al., 1993). In biofilms and sediments receiving high levels of oxidizable organic matter, oxygen penetration depth can be as small as 100 μm and sometimes even almost 0 μm (De Beer, 1990; cited in Kato et al., 1993). Similarly, in aerobic granules containing glycogen-accumulating bacteria oxygen did not penetrate further than 100 μm during 2 hr of oxygen exposure (Meyer et al., 2003).

This shielding effect (habitat segregation) has been used to develop a co-immobilized mixed culture system of aerobic and anaerobic microorganisms in calcium alginate beads under aerobic conditions. The aerobic microorganisms grew in the oxygen-sufficient surface area, while anaerobic ones grew mainly in the oxygen-deficient central part of the gel beads (Figure 2.2). The co-immobilized mixed culture system also acted jointly to produce fermented products such as ethanol and lactic acid from starch through aerobic and anaerobic metabolic pathways (Kurosawa and Tanaka, 1990).

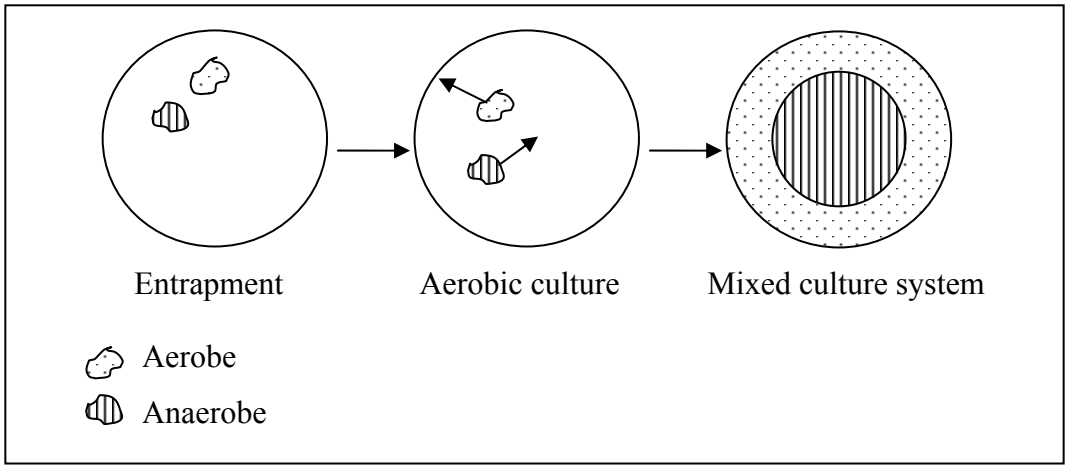


Figure 2.2. Behaviour of anaerobes and aerobes in co-immobilized mixed cultures system (Kurosawa and Tanaka, 1990).

The shielding effect on the survival of methanogenic bacteria can be explained in mixed cultures by the role of facultative bacteria. For instance, facultative microorganisms on the surface of granular sludge particles from an UASB reactor consumed oxygen before it diffused into the inner-particle region. Thus, these microorganisms could potentially protect intolerant methanogens from exposure to oxygen. The 50% inhibiting concentration (IC_{50}) of various samples of anaerobic granular sludge exposed to oxygen for three days ranged from 7 to 41% O_2 in the head space at the start of the exposure period. These values corresponded to DO concentrations ranging from 0.05 to 6.1 mg/L in the bulk liquid phase at the end of the exposure period (Kato et al., 1993). IC_{50} values were found to be highly correlated to specific oxygen uptake rates of the sludges tested which clearly indicates that consumption of oxygen was a major factor in the remarkably high tolerance to oxygen. Considering that penetration of oxygen in active biofilms would only be a hundred micrometers and that the granules had diameters ranging from 1.3 to 2.8 mm, voluminous anaerobic zones were most likely present inside the granules where the methanogenic bacteria were protected from contact with oxygen.

The shielding effect was even used to improve the treatment efficiency of anaerobic systems. Bench-scale batch reactor studies were also performed with a mixed culture of anaerobic digester sludge and aerobic mixed liquor under oxygen-limited conditions (Zitomer and Shrout, 1998). Two oxygen addition rates, namely 1 and 0.1 g O_2/L.day, were applied. Organic loading rate in terms of chemical oxygen demand (COD) ranged from 0.25 to 4 g/L.day. COD removal efficiencies of the two oxygen-limited reactors and the strictly anaerobic and aerobic control bench-scale reactors receiving 30 g/L sucrose were greater than 93%. The system receiving 1 g O_2/L.day achieved a lower final effluent COD than strictly anaerobic reactor. After shock-loading was applied to investigate the process stability and recovery, pH recovered in oxygen-limited reactors, whereas pH in the anaerobic reactors did not recover. It is possible that under aerobic (or

oxygen-limited) conditions, stripping of CO_2 and H_2 increased the rate of pH recovery in the systems under low aeration.

The tolerance of anaerobic cultures to the microaerobic conditions also appears as an alternative approach for the treatment of wastewaters with high COD and high sulfate (SO_4^{-2}) concentrations. Although anaerobic biological treatment may be an economical method for COD reduction, SO_4^{-2} is typically converted by sulfate-reducing bacteria to hydrogen sulfide (H_2S), which is inhibitory to methane-producing bacteria, slowing or stopping methane productions at higher concentrations greater than approximately 50 to 100 mg H_2S/L (Zitomer and Shrout, 2000). Recently, limited aeration of recycle flow to hybrid and baffled reactors has been used to treat this wastewater and shown to reduce aqueous H_2S concentrations by causing production of uninhibitory sulfur (S^0) and thiosulfate ($S_2O_3^{-2}$) as well as gas stripping volatile H_2S. It has been reported that COD:SO_4^{-2} is an important measure of treatability and with a ratio less than approximately 4:1 indicating potential toxicity problems related to H_2S production (Speece, 1996). Zitomer and Shrout (2000) investigated the treatment of high sulfate and high COD wastewater using aerated methanogenic fluidized bed reactors (FBR) with anaerobic digester sludge as seed culture. Organic loading rates of 8-50 g COD/L.day and influent sulfate concentrations of 160-1200 mg/L were applied to the reactors to determine the effects of shock loads of COD and sulfate. The aeration rate ranged from 60 to 675 mL air/min. It was observed that directly aerated methanogenic FBRs achieved increased methane production and COD removal (87%) compared to strictly anaerobic FBRs treating high-sulfate wastewater (COD removal, 27%). Oxygen transfer satisfying 28% of the COD load resulted in maximum SOUR of 0.20 mg O_2/g VSS.min (milligram oxygen per gram volatile suspended solids per minute), with significant methane production. Considering the SOUR of a pure aerobic *Eschericchia coli* and municipal activated sludge of 0.21 mg O_2/g VSS.min and 0.17-0.33 mg O_2/L.min, respectively, it was concluded that mixed methanogenic cultures can use a significant mass of oxygen and produce methane. Under typically inhibitory SO_4^{-2}

loading, higher aeration caused increased effluent SO_4^{-2}, increased H_2S mass in the off-gas and lower H_2S concentration. COD removal and methane production increased with increasing aeration rate due to the decreased concentration of H_2S in more aerated reactors. Air addition also results in lower alkalinity supplementation to maintain neutral pH because acidic CO_2 is also stripped from the reactor. The associated pH increase caused by CO_2 stripping shifts sulfide speciation to less toxic HS^- and therefore, is another benefit of aeration.

2.1.1.2. Intrinsic Tolerance to Oxygen

Aerobic and facultative bacteria are regarded as possessing appropriate protective mechanisms against the oxygen radical so far. The main hypothesis for the protective mechanisms is the ability to produce two enzymes, catalase and superoxide dismutase (SOD) which is an oxygen defense enzyme of the aerobes neutralizing toxic oxygen radicals. SOD seems indispensable to all the aerobes, despite the claim that a few aerobes lack it. The total lack of SOD has also been suggested as the reason for oxygen intolerance among strict obligate anaerobes. Curiously enough, many obligate anaerobes do contain SOD and they can tolerate to some extent low levels of direct contact with oxygen (Rolfe et al., 1978). These findings reveal that the obligate anaerobes differ in their sensitivity to oxygen, varying from those strict intolerance to others possessing some intrinsic tolerance, which might explain their survival when exposed to low levels of oxygen.

The most noteworthy group of strict anaerobic bacteria involved in wastewater treatment processes are the methanogens. Since the early works on the isolation of pure cultures of *Methanosarcina barkeri* and *Methanobacterium formicicum*, the widespread view is that the methanogens are fastidious microorganisms requiring strict anaerobic conditions for growth and methane production. Possible traces of oxygen are eliminated by the addition of reducing agents such as cysteine or sulphide, to poise the redox potential within the low range. Indeed many

methanogens are acutely sensitive to oxygen. *Methanococcus voltae* and *Methanococcus vannielii* were shown to be highly intolerant to oxygen, which corresponded to their lack of SOD (Kiener and Leisinger, 1983). Also *Methanobacterium ruminantium*, *Methanobacterium mobile* and *Methanobacterium* sp. strain AZ were also found to be highly sensitive to oxygen since their growth and methane production were completely prevented at DO concentration of 0.01 mg/L (Zehnder and Wuhrmann, 1977).

However, other investigations indicate that at least some methanogens do have intrinsic tolerance to oxygen. For instance, oxygen tolerance was observed among several species of bacteria in the order Methanomicrobiales and *Methanobacterium bryantii* and was attributed to SOD. *Methanobacterium thermoautotrophicum* and *Methanobrevibacter arboriphilus* and *Methanosarcina barkeri* strains, which were originally isolated from sludge digesters, survived for hours in the presence of air without decrease in the number of colony forming units (Kiener and Leisinger, 1983). Enrichment cultures of *Methanothrix soehngenii* derived from anaerobic sludge digesters were exposed to pure oxygen for 48 hours. The exposure did not cause cell lysis or loss in methane production (Huser et al., 1982).

It has also been demonstrated that strictly aerobic and anaerobic bacteria can grow side by side under microaerobic conditions in chemostat cultures provided the supply of oxygen does not exceed the maximum O_2 consumption of the aerobic bacteria. Under such oxygen-limited conditions, fermentative, sulfate-reducing and even very oxygen-sensitive methanogenic bacteria have been shown to coexist with obligately aerobic bacteria (Gerritse et al., 1990).

The feasibility of methanogenesis under oxygen-limited conditions was investigated by Zitomer and Shrout (1998). Serum bottle studies were performed with mixed cultures from anaerobic digesters, which were acclimated by a 2-day wasting/feeding cycle and oxygenation with 4, 12 and 48 mL of pure oxygen (10,

30 and 125% of the total COD added (50 mg sucrose) for 30 days. Methanogenic activity of the serum bottle cultures was examined after withholding of oxygen from the bottles, addition of acetate as carbon source and measuring the daily gas production for the consecutive 7 days. Other electron acceptors like sulfate and nitrate were negligible. It was found that maximum biomass yields of oxygen-limited cultures ranged from 0.13 to 0.07 g VSS/g sucrose as COD and are more typical of strictly methanogenic rather than aerobic processes. Oxygen addition did not prevent the growth of methanogens, but increased their initial activity under studied conditions. The absence of large flocs or pellets but the predominance of free-swimming bacteria in the bottles indicates that the survival of methanogens under low-aerated conditions was not due to formation of anaerobic microniches but was probably due to the intrinsic tolerance of methanogens. Higher COD removals and lower effluent COD concentrations were even exhibited under oxygen-limited conditions.

Aside from the intrinsic tolerance due to the SOD that some methanogenic species possess, other factors have been postulated to be involved in protection against oxygen. *Methanosarcina barkerii* strain Fusaro was shown to have a number of redox carriers decreasing the redox potential when chemical oxidant agents were used. However, this reducing capacity was not enough to avoid the inhibition by oxygen at concentrations higher than 0.5% in the gas phase. Nonetheless, the capacity to adjust the redox potential in its own redox environment may partly explain good survival in dry and oxic soil (Fetzer and Conrad, 1993).

2.1.2. Applications of Aerobic and Anaerobic Cultures Co-existing in the Same Environment

The survival of anaerobic cultures under aerobic or microaerobic (DO<1 mg/L, Yerushalmi et al., 2001) conditions due to either intrinsic tolerance or formation of anaerobic niches (shielding effect) guided the studies where two different types

of cultures, i.e. anaerobes and aerobes located in the same environment / reactor. Considering the location of the cultures in the reactor, studies can be classified as follows:

- Both anaerobic and aerobic species co-immobilized in natural polymers such as (calcium alginate) gel beads operating under aerobic and/or microaerobic conditions (Beunink and Rehm, 1988, 1990; Kurosawa and Tanaka, 1990; Meyerhoff et al., 1997).
- Anaerobic cultures embedded into supportive media (e.g. nylon netting) or granular anaerobic cultures and suspended aerobic cultures placed in a reactor operating under aerobic/microaerobic conditions (Gerritse and Gottschal, 1992; Gardin et al., 2001).
- Suspended anaerobic and aerobic cultures in the reactor operating under microaerobic/oxygen-limited conditions (Gerritse et al., 1990; Zitomer and Shrout, 1998).
- Mixed suspended anaerobic (or anoxic) and aerobic cultures in packed-bed bioreactors (Hutchins et al., 1992; Boopathy et al. 1998; Yerushalmi et al., 1999).

In all these studies with either free or co-immobilized cultures of anaerobes (or anoxic cultures) and aerobes, it is observed that DO concentrations display alternating values. In other words, oxygen gradient results in alternating conditions from aerobic and/or microaerobic to anaerobic conditions either through the reactor content (as in packed-bed or slurry reactors) or from bulk liquid to the depths of the immobilized co-cultures. The use of cultures requiring different environmental parameters such as oxygen levels makes this co-existence possible (O'Reilly and Scott, 1995). The possible applications of the co-existence of anaerobic/anoxic and aerobic cultures in the same reactor might present significant opportunities within the environmental engineering field, as discussed below.

Immobilization of cells in natural polymers such as Ca-alginate, agar and K-carrageenan, has been widely used for the production of biochemicals, mainly because the method is considered to be inexpensive and easy (Kurosawa and Tanaka, 1990). However, this technique of entrapping cells in a polymer gel matrix poses a problem of mass transfer resistance by the gel matrix. In particular, oxygen often becomes a limiting factor in aerobic fermentation with immobilized cells. The formation of anaerobic zones in these gel matrix or aerobic biofilms was accepted as a disadvantage. However, utilization of the oxygen-deficient part as the growth area for anaerobic cells is simpler than improving the oxygen supply to the gel bead or biofilms (Kurosawa and Tanaka, 1990). In other words, this disadvantage can now be used to promote the complete degradation of typical recalcitrant xenobiotic aromatic compounds. Many recalcitrant aromatic compounds that are difficult to degrade aerobically seem to be readily biotransformed anaerobically. In turn, the products of the anaerobic transformation resist further anaerobic biotransformation; yet they are good substrates for aerobic biodegradation. Thus for the total mineralization of many recalcitrant pollutants, a sequenced anaerobic-aerobic treatment strategy should be sought. The anaerobic zones that develop in the biofilms can therefore be used to house microorganisms displaying anaerobic activity towards the reduction of these xenobiotics compounds. Concomitantly, microorganisms in the aerobic zones of the gel beads or biofilms can be used to mineralize the reduced products, enabling the benefits of sequenced anaerobic-aerobic treatment in one reactor (Field et al., 1995). There are examples in literature where the recalcitrant behavior of aromatic pollutants was overcome on co-cultures constructed from anaerobic and aerobic bacteria (Beunink and Rehm, 1988; 1990; Gerritse and Gottschal, 1992; Field et al., 1995; Gardin et al., 2001).

Beunink and Rehm (1988) used an anaerobic-aerobic co-culture for the degradation of a mixture of DDT and 4,4'-dichlorodiphenlymethane (DDM). A DDT dechlorinating bacterium, *Enterobacter cloacae*, isolated from digested sewage sludge was co-immobilized into calcium alginate beads together with

Alcaligenes sp. strain Iso DPM4, an obligate aerobic bacterium that was able to cometabolize DDM with diphenlymethane as a primary substrate. The co-culture placed into an aerated fermentor, where the bulk liquid phase DO concentration ranged from 2.4 to 8 mg/L, was able to simultaneously dechlorinate DDT and mineralize DDM. That dechlorination was possible under the aerobic conditions prevailing, was attributed to the anaerobic microniches in the center of the gel beads.

The coupled reductive and oxidative degradation of 4-chloro-2-nitrophenol (CNP) was investigated in an aerated airlift fermentor using a co-culture system obtained by immobilizing facultative anaerobe *Enterobacter cloacae* and obligate aerobe *Alkaligenes* sp. strain TK-2 in Ca-alginate beads (Beunink and Rehm, 1990). The previous degradation studies applied with single cultures demonstrated that *Enterobacter cloacae* was able to reduce CNP to 4-chloro-2-amoniphenol (CAP) and 4-chloro-2-acetaminophenol (CAAP) under anaerobic conditions, while unable to metabolize CAP in either anaerobic or aerobic conditions. On the other hand, *Alkaligenes* sp. strain TK-2 was able to mineralize CAP under aerobic conditions, while unable to metabolize CNP. However, with the co-immobilized cells where *Enterobacter cloacae* and *Alkaligenes* placed in the anaerobic zone and the aerobic outer periphery of the bead, respectively, CNP was completely mineralized to CO_2, Cl^- and NH_4^+. Thus, the complete mineralization can be rationalized by the migration of CNP into the anaerobic zone and subsequent migration of the anaerobic products CAP and CAAP to the aerobic zone.

Reductive dechlorination of a persistent herbicide 2,3,6-trichlorobenzoic acid (2,3,6-TBA) was studied with a co-culture of anaerobic-aerobic bacteria in a chemostat under microaerobic conditions (Gerritse and Gottschal, 1992). An anaerobic enrichment culture derived from fresh water sediments, which reductively dechlorinate 2,3,6-TCA to 2,5-dichlorobenzoic acid (2,5-DBA), was immobilized on vermiculite (a clay mineral abundant in soils) support placed in nylon netting. A 2,5-DBA mineralizing obligate aerobe *Pseudomonas aeruginosa*

JB2 was also introduced into the reactor. While the anaerobic enrichment culture was unable to further dechlorinate 2,5-DBA and *Pseudomonas aeruginosa* in pure culture was unable to degrade 2,3,6-TBA, the aerated co-culture completely mineralized 2,3,6-TBA, with 95% recovery of organochlorines as Cl^-. Reductive dechlorination of 2,3,6-TBA to 2,5-DBA was accomplished by anaerobic culture in the immobilized biomass and further mineralization carried out by free suspended cells of *Pseudomonas aeruginosa* in the bulk liquid phase. The DO concentration in the reactor bulk liquid ranged from 9.6 to 16 μg/L, while the redox potential readings ranged from +134 to +299 mV. CH_4 production observed under anaerobic conditions before aeration of the reactor and subsequent addition of aerobic bacterium dropped significantly. However, dechlorination was not significantly inhibited by addition of air, probably due to the shielding effect of aerobic bacteria and formation of suitable growth conditions for anaerobes in the nylon bag with vermiculite. In addition, despite of the microaerobic conditions in the reactor, *Pseudomonas aeruginosa* maintained a high cell-density to oxidize all 2,5-DBA due to the availability of additional organic substrates such as benzoate, yeast extract, peptone and fermentation products of anaerobic population.

Tricholorophenols (TCPs) are widely used as wood preservatives, fungicides or precursors in the synthesis of phenoxyacetate herbicides and they have contaminated numerous soil and groundwater environments. In anaerobic conditions, multichlorophenols are usually degraded by reductive dehalogenation and transformed into mono- and dichloro-phenols which remain in the environment because of their low degradation rates. On the other hand, these mono- and dichloro-phenols can successfully be degraded by aerobic catabolism through the oxidation of the aromatic ring. Gardin et al. (2001) studied the degradation of 2,4,6-TCP in a single reactor with co-immobilized anaerobic microorganisms (in gel beads) and a selected mixed aerobic community in an USB reactor. They achieved complete mineralization of 2,4,6-TCP under air-limited conditions (36-48 vvd, volume of air/volume of liquid in the reactor per day) indicating the coupled reaction of both cultures.

Groundwater contamination by gasoline and its hydrocarbon constituents leaking from underground storage tanks, distribution systems and various industrial operations is a major environmental problem. Conventional treatment techniques such as pump and treat and air stripping have limited applications and efficiencies due to their high cost and maintenance requirements and transfer of contaminants from one phase to another. In-situ treatment techniques, which include the mechanisms of sorption, oxidation/reduction and chemical or biological degradation, have considerably lower costs and maintenance requirements compared to ex-situ techniques. However, under in-situ conditions, there is limited supply or complete lack of oxygen, or in other words, oxygen-limited or anoxic conditions usually prevail. Thus, oxygen limitation is one of the major problems affecting the performance of in-situ biological treatment systems (Yerushalmi et al., 1999). Also, supplying an alternative electron acceptor such as sulfate and nitrate is more economical and more convenient. Considering the oxygen-limited conditions prevailing under in-situ treatment techniques, Yerushalmi et al. (1999) investigated the continuous bioremediation of gasoline-contaminated water in a microaerophilic packed-bed biobarrier system. The gasoline sample contained 28.9% BTEX (benzene, toluene, ethylbenzene and xylenes) which are the most soluble aromatic hydrocarbons in gasoline. The inoculum was an enrichment culture of an indigenous microbial from a soil sample. The inlet gasoline concentrations ranged from 3.7 to 74 mg/L. Although the inlet DO concentration was 8 mg/L, the values measured throughout the reactor and in the effluent were less than 0.3 mg/L, indicating the quick consumption of oxygen and formation of oxygen-limited (microaerophilic) condition in the reactor. The high gasoline removal efficiencies (>99%) were achieved with limited supply of molecular oxygen (less than the demand for complete oxidation of gasoline) implying that microaerophilic conditions did not prevent the degradation of gasoline. The consumption of sulfate and the presence of sulfate-reducing bacteria in the biobarrier suggested the presence of anaerobic metabolism during degradation of gasoline. Two mechanisms were proposed for

biodegradation of gasoline hydrocarbons; first, the contribution of anaerobic metabolism, implying an alternative electron acceptor in the system in addition to oxygen, and second, the aerobic transformation of hydrocarbons into intermediate metabolites. The remaining fraction of gasoline in the effluent consisted mainly of three aliphatics, and not the aromatic compounds.

Based on the reports indicating the incomplete degradation of 2,4,6-trinitrotoluene (TNT) by aerobic bacteria alone or sulfate-reducing bacteria under anaerobic conditions alone, Boopathy et al. (1998) investigated the use of aerobic/anoxic soil slurry reactor technology for removal of TNT from contaminated soil. The reactor was operated semi-continuously, molasses was added as the carbon source and air was supplied once a day for 10 minutes through a diffuser. An oxygen profile was observed ranging between 4 to 0 mg/L through the reactor. The radio-labeled TNT incubated with reactor biomass showed that 23% of [^{14}C] TNT was mineralized, 27% was converted to biomass and 8% was adsorbed onto soil. The rest of the [^{14}C] TNT was accounted for as metabolites, including ring cleavage product identified as 2,3-butanediol.

Another treatment application of co-culture system (with aerobic and anoxic cultures) is for the combined nitrification and denitrification in a single reactor under microaerobic conditions. Biological processes for nitrification with denitrification are becoming very important for wastewater treatment. The addition of denitrification to a nitrification process has several advantages over nitrification alone in terms of complete nitrogen removal and reduced aeration requirement, which saves running costs. Watanabe et al. (1995) investigated the simultaneous nitrification with denitrification using a single rotating biological contactor (RBC) under microaerobic conditions. The combination of nitrification and denitrification can occur only under the following conditions; nitrifiers and denitrifiers must be present in the biofilm, and suitable growth conditions for each of the responsible strains of bacteria must be created in the biofilm. The primary control parameter is oxygen transfer rate, which must be sufficient to support

nitrification and aerobic oxidation of organic matters and low enough to satisfy denitrification. The evidence suggests that simultaneous nitrification with denitrification could be achieved by controlling the oxygen partial pressure (P_{O2}) in the air phase. Second control parameter is spatial distribution of bacteria and the third one is the type of organic source and its concentration relative to nitrogen concentration. It was observed in this study that, simultaneous nitrification with denitrification in a single reactor could be enhanced by reducing the P_{O2} in the air phase. The maximum nitrogen removal efficiency of 90% was obtained at around P_{O2} of 0.10 atm and a hydraulic loading of 0.7 g NH_4-N/m^2.day. Using a microslicer technique, microbial spatial distribution in the biofilm was determined and nitrifiers, denitrifiers and heterotrophs were found to coexist throughout the biofilm. This indicated that combined nitrification and denitrification is possible where the local environment conditions meet their growth. An experiment with a combined partially and fully submerged RBC (CPFSR) reactor was also carried out to investigate the effects of organic source type and influent carbon:nitrogen (C/N) ratio on the efficiency of combined nitrification and denitrification system. CPFSR reactor presented better performance of simultaneous nitrification with denitrification at C/N ratios of 2, 4 and 6 over a partially submerged RBC alone, because nitrifying biofilms were mainly developed on partially submerged disks, while denitrifying biofilms were developed on fully submerged disks. Poly-vinly-alcohol (PVA) was degraded and used for denitrification as a carbon source.

In addition to the combined nitrification / denitrification, complete mineralization of recalcitrant xenobiotic compounds (and aromatics) such as chlorobiphenlys, chlorophenols, compounds with nitro substituents, the applications of mixed anaerobic and aerobic cultures in the same reactor can be extended for the treatment (decolorization and biodegradation) of azo dyes and enhanced biological phosphorous removal. Mineralization of chloroethylenes (such as percholorethylene, PCE) is also a potential application area for co-cultures of anaerobes and aerobes. PCE, a suspected carcinogen, is known to be a common groundwater pollutant, thus its removal from contaminated sites is significant.

Degradation of PCE occurs only under anaerobic conditions, while more readily degradation of less-chlorinated ethylenes under aerobic conditions. By mixed anaerobic and aerobic cultures, the complete mineralization of PCE requiring sequential anaerobic and aerobic treatment can be achieved in one reactor.

As mentioned previously, the systems (reactors) containing both anaerobic and aerobic cultures (coupled reactors) might be advantageous compared to the conventional anaerobic and aerobic treatment systems. Aeration and oxygen-limited conditions resulted in decreased alkalinity requirement due to CO_2 stripping, increased COD removal, pH recovery and methane production compared to anaerobic systems (Zitomer and Shrout, 1998; 2000). The large amount of excess sludge problem in the conventional activated sludge systems can also be addressed in the coupled reactors via the existence of slowly-growing anaerobic cultures. This also means reduced cost for the handling, transport and disposal of produced biosolids.

Coupling the aerobic cultures with anaerobic ones under aerobic/microaerobic conditions or alternating anaerobic/aerobic conditions might be also more energy efficient than conventional aerobic systems due to lower aeration requirement and additional methane production. Many individual electron donors in a mixture are stated to be oxidized concurrently not sequentially. The highest free-energy releasing electron acceptor is not always employed exclusively or first. Besides, methane might be also produced sequentially by CO_2-consuming methanogens using the end product of aerobic degradation (i.e. CO_2) under aerobic conditions. Zitomer (1998) reported concurrent aerobic respiration and methane production under oxygen-limited conditions. Methane constituted the 46.6 and 15.2% of the gas composition in the reactors fed with oxygen doses of 10 and 30% of the total COD added (25 mg or 500 mg/L.day), respectively. Shen and Guiot (1996) also indicated the methane production under microaerobic and aerobic conditions. Anaerobic granules exposed to influent COD concentrations of 6.06-7.06 g/L and DO concentrations of 0.5, 2, 5 and 8 mg/L for one month produced 2.9-3.8 L

CH_4/day with CH_4% values of 8.3 to 27.1% increasing with the decreasing DO concentrations.

Even if the methane portion in the gas composition of mixed anaerobic and aerobic cultures in a reactor might be as low as 1%, it might still be used beneficially. USEPA (2003) identified two technologies for destroying or beneficially using the methane contained in ventilation air: a thermal flow reversal reactor (VOCSIDIZER) and a catalytic flow-reversal reactor developed expressly for mine ventilation air. Both technologies employ similar principles to oxidize methane contained in mine ventilation airflows. Based on laboratory and field experience, both units can sustain operation (i.e. can maintain oxidation) with ventilation air having uniform methane concentrations down to approximately 0.1 percent. For practical field applications where methane concentrations are likely to vary over time, however, this analysis assumes that a practical average lower concentration limit at which oxidizers will function reliably is 0.15 percent. In addition, a variety of other technologies such as boilers, engines and turbines may use ventilation airflows as combustion air. At least two other technology families may also prove to be viable candidates for beneficially using ventilation air methane. These are VOC (volatile organic compound) concentrators and new lean-fuel gas turbines (USEPA, 2003).

Methane might be explosive in low concentrations (4.36 to 15.55 percent in air). For concentrations below the lower explosive limit (LEL, 4.36%) the mixture is too lean to burn and for concentrations above the upper explosive limit (UEL, 15.55%) the mixture is too rich to burn (De Nevers, 1995). The possible problem in coupled reactors can be overcome by arrangement of the cyclic operations; mixed cultures might be exposed to cyclic anaerobic and aerobic conditions to prevent the explosion possibility.

The other application field of mixed anaerobic and aerobic cultures might be the granulation technology. Anaerobic/anoxic zones in the activated sludge systems

(Wu et al., 1987; Noyola and Moreno, 1994; Lens et al., 1995) and in aerobic granules (Yu and Bishop, 1998; Tay et al., 2002a) were reported in literature. These zones can be more effectively used in coupled reactors via anaerobic cultures. Especially granulation of anaerobic and aerobic cultures (in the same reactor) appears to be promising for effective use of anaerobic zones by location of anaerobic cultures in the inner oxygen-free parts. Despite of the advantages of treatment with granular cultures, granulation by using these two cultures has not been studied so far except by Ferguson (1999) who investigated the PCE and benzene degradation but observed pelletization. The studies were limited to the co-immobilization of specific anaerobic and aerobic cultures (not mixed cultures) in gel beads (Beunink and Rehm, 1988; 1990; Kurosawa and Tanaka, 1990; Meyerhoff et al., 1997).

Despite of the great interest little is known about the regulation mechanisms and applications of mixed anaerobic and aerobic cultures. Knowledge of microbes exposed to an anaerobic-aerobic sequence is also lacking (Zeng and Deckwer, 1996). Recently, aerobic granulation studies, which were usually studied under aerobic (Mongenroth et al., 1997; Beun et al., 1999; Etterer and Wilderer, 2001; Wang et al., 2004; Hu et al., 2005a) or microaerobic (Peng et al., 1999; Hu et al., 2005b) conditions, have been performed under alternating anaerobic (or anoxic) and aerobic conditions for complete nitrogen removal (Yang et al., 2003; Jang et al., 2003). However, exposure of the anaerobic and aerobic cultures co-existing in the same reactor to an anaerobic-aerobic sequence has not been studied so far. Alternating cyclic conditions might play significant role for the survival of suspended mixed anaerobic and aerobic cultures co-existing in the same environment.

As a result, the use of anaerobic and aerobic cultures co-existing in one reactor (i.e. in coupled reactor) appears to be promising for improving the efficiency and reducing the costs compared to the conventional anaerobic or aerobic treatment systems, however it is still in its infant stage. Being investigated recently, the

anaerobic (or anoxic) and aerobic cultures used in the coupled reactors have not been clearly defined. Some of the definitions used for these cultures are given in Table 2.1.

Table 2.1. Some of the definitions used for the anaerobic (anoxic) and aerobic cultures co-existing in the same reactor.

Reference	Conditions	Seed sludge	Culture named as
Gardin et al., 2001	Air-limited	Anaerobic granular sludge + aerobic suspension	Co-existing anaerobic and aerobic communities under air-limited conditions
Zitomer and Shrout, 1998	Oxygen-limited	Anaerobic digester sludge + aerobic mixed liquor	Oxygen-limited cultures
Yerushalmi et al., 1999	Micro-aerophilic	Enriched cultures from top layer of gasoline-contaminated soil.	Not defined
Gerritse and Gottschal, 1992	Low / limited aeration	Suspended aerobic + nylon bag consisted of anaerobic bacteria	Mixed cultures of anaerobic and aerobic bacteria operating under oxygen-limitation
Gerritse et al., 1990	Oxygen-limited	Methanogenic culture + microaerobic chemostat culture + strict aerobic cultures	Mixed cultures growing in one habitat under oxygen-limited conditions
Kurosawa and Tanaka, 1990	Aerobic	Aerobe + obligate anaerobe and/or aerobe + facultative anaerobe	Co-immobilized mixed culture system

As seen in Table 2.1, a common name has not been used. Therefore, considering the scope of the thesis, the anaerobic and aerobic cultures that survived and operated in the same reactor under alternating anaerobic / aerobic and/or microaerobic conditions were defined by the term ‘combined cultures’.

2.2. Granulation

Granules are dense microbial consortia packed with different bacterial species and typically contain millions of organisms per gram biomass. They form through self-immobilization of microorganisms without relying on the need for carriers or artificial surfaces for cell attachment (Liu and Tay, 2004). Granules are developed via a multiple-step process that involves a variety of physicochemical and biological forces (Liu and Tay, 2002, 2004). Flocs, which are also forms of cell immobilization, are most commonly associated with the well-known activated sludge process that was discovered more than 100 years ago. However, granules are a more recent discovery that was first reported in UASB systems (Lettinga et al., 1980). Compared with sludge flocs of loose structures, granules have well-defined denser, firmer and more compact structures (Schmidt and Ahring, 1996).

For many years studies have focused on anaerobic granulation, which is first and best recognized in UASB reactors. Every granule is a biological treatment unit with mixed cultures. By means of anaerobic granules, UASB reactors can retain high biomass concentrations in the presence of upflow wastewater velocity and biogas production. Good settleability, high biomass concentration (30000 to 80000 mg/L) and excellent solid-liquid separation are realized with proper granulation in UASB reactors. Organic and hydraulic loading rates of 30-40 kg COD/m^3.day and 8 m^3/m^3.day, respectively and even higher rates are possible. High concentration and granular structure of biomass ensure that the performance of UASB is not readily disturbed by sudden high loadings and toxic shocks compared to the suspended growth systems (Maat and Habets, 1987; Speece,

1996). Anaerobic granular sludge has therefore proved to be capable of treating high-strength wastewaters (Liu and Tay, 2004). Almost all granulation research has been carried out in UASB reactors. But a few anaerobic granulation study performed in internal circulation (IC) reactor, anaerobic sequencing batch reactors (SBRs), anaerobic migrating blanket reactor and anaerobic continuous stirred tank reactors were demonstrated (Angenent and Sung, 2001; Liu and Tay, 2004).

In fact, granulation is not only restricted to anaerobic cultures. Granulation by acidifying bacteria (Beeftink, 1987; cited in Beun et al., 1999) and nitrifying bacteria (De Beer et al., 1993) have also been reported. Over the past few years, research attention had turned towards developing aerobic granular sludge (Tijhius et al., 1994; Morgenroth et al., 1997; Tay et al., 2001a, 2001b; Beun et al., 2002; Liu et al., 2004; Tay et al., 2004a; 2004b). As compared with conventional activated sludge flocs, the advantages of aerobic granular sludge are the regular, denser and stronger microbial structure, good settling ability, high biomass retention, less vulnerability to toxic organic chemicals and ability to withstand a high organic loading rate as the anaerobic granules (Tay et al., 2001b; Liu and Tay, 2004). Studies indicated that granules of aerobic heterotrophic microorganisms have been mostly cultivated in SBRs (Morgenroth et al., 1997; Beun et al., 1999; Etterer and Wilderer, 2001) as well as in sequencing batch airlift reactors (Beun et al., 2000).

The granule size is an important parameter in the characterization of aerobic granulation. The average diameter of aerobic granules varies in the range of 0.2-5 mm (Liu and Tay, 2004) similar to that of anaerobic granules as 0.14-5 mm (Schmidt and Ahring, 1996). The settling velocity of the aerobic granules is associated with the granule size and structure and is as high as 22-70 m/hr (Morgenroth et al., 1997; Liu and Tay, 2004; Hu et al., 2005a). This is comparable with those of anaerobic granules given as 20-50 m/hr (Schmidt and Ahring, 1996) and 30-94 (Batstone and Keller, 2001), but at least three times higher than those of activated sludge flocs (around 8-10 m/hr).

Despite of many advantages, both anaerobic and aerobic granulation technologies have some drawbacks. Anaerobic granulation needs a long start-up (2-4 months or longer, Lettinga et al., 1980; Liu et al., 2003a; Liu and Tay, 2004), a relatively high operation temperature and unsuitability for removal of nutrients (N, P) (Liu and Tay, 2004). On the other hand, compared to anaerobic granules, aerobic granules have relatively low stability because of their fast-growth rate. In addition, due to the diffusion limitation of oxygen and nutrients into the granule interior, the aerobic cultures forming the granules cannot be fully utilized and marked deterioration in granule activity is observed (Tay et al., 2002b; Liu and Tay, 2004).

2.2.1. Factors Affecting Granulation

The ability to form granules depends on many environmental and operational factors including the seed-sludge characteristics (such as methanogenic activity, sludge concentration and settleability), feed composition, hydraulic/organic loading rates, hydraulic retention time (HRT), upflow velocities, substrate type, temperature and pH.

Characteristics of the feed (substrate type) is considered as a key factor influencing the formation, composition and structure of the anaerobic granules (Schmidt and Ahring, 1996). Aerobic granule microstructure and species diversity also appear to be related to the type of carbon source (Liu and Tay, 2004). Granulation has been successfully achieved with a wide variety of substrates such as ethanol, glucose, acetate for aerobic cultures (Beun et al., 1999; Peng et al., 1999; Tay et al., 2001a; Hu et al., 2005a) and with ethanol, glucose, acetate, propionate, butyrate, maize processing waste, brewery for anaerobic cultures (Ross, 1984; Dolfing et al., 1985; Grotenhuis et al., 1991; O'Flaherty et al., 1997; Batstone and Keller, 2001). Based on the free energy of oxidation, organic

substrates can be roughly classified into high-energy (e.g. glucose and sucrose) and low-energy substrates. During anaerobic granulation start-up period, high-energy feeding can sustain the acidogens and facilitate the formation of extracellular polymers (ECPs). The presence of high-energy substrates and consequently higher concentrations of acidogens are essential for a well-granulated anaerobic biomass. The more readily the acidogens metabolize the substrate, the more rapidly the protons will be activated and sooner the methanogens will obtain the substrate (Tay et al., 2000). On the other hand, the type of carbon source is insignificant for aerobic granulation period due to the fast growth of aerobic bacteria (Liu and Tay, 2004).

Organic loading rate (OLR) and sludge loading rate (SLR, kg of carbon source loaded per kg VSS per day) have an essential role in anaerobic granulation. The operating start-up parameters used / recommended in literature for achievement of granules from suspended anaerobic cultures in UASB reactors are summarized in Table 2.2. Anaerobic granulation can be accomplished by gradually increasing space OLR (SOLR) from 1 to 9 (even 12.5) kg COD/m^3.day, or gradually increasing SLR from 0.1 to 0.6 (even 1) kg COD/kg VSS.day (Lettinga and Hulshoff Pol, 1986; Wu et al., 1987; Noyola and Moreno, 1994; Ghangrekar et al., 1996; Yu et al., 2001). However, aerobic granulation is not sensitive to the OLR. Aerobic granules can form across a very wide range of OLR ranging from 2.5 to 15 kg COD/m^3.day. The mean size of the granules may increase with the increased OLR. However, too high OLR, on the other hand, results in the decrease of the strength of both anaerobic and aerobic granules (Quarmby and Forster, 1995; Tay et al., 2001a, 2001b; Liu and Tay, 2004).

Shear force favors the formation of both anaerobic and aerobic cultures. Hydrodynamic shear force for anaerobic granulation is achieved by liquid upflow velocity in UASB reactors. High liquid upflow velocity and short HRT support the anaerobic granulation by leading to the washout of the non-granulation competent (O'Flaherty et al., 1997; Liu and Tay, 2004). It is stated that increased

liquid upflow velocity increases the settling property and sludge volume index (SVI) of the anaerobic granules (Noyola and Moreno, 1994). Liquid upflow velocities as low as 0.03-0.04 m/hr (Speece, 1996) to 0.5 m/hr (Lettinga and Hulshoff Pol, 1986) can be applied in UASB reactors for anaerobic granulation. In aerobic granulation, shear force is achieved by superficial upflow air velocity. Tay et al. (2001a) stated that aerobic granules could be formed only above a threshold shear force value in terms of superficial upflow air velocity above 1.2 cm/s in a column SBR. The granule density and strength are highly dependent on shear force applied. They also reported that the production of extracellular polysaccharides was closely associated with the shear force. High shear force stimulated the bacteria to secrete more extracellular polysaccharides and thus more compact and stronger aerobic granules.

Table 2.2. UASB start-up parameters used / recommended in the studies.

Influent COD* (g/L)	HRT* (hr)	Upflow velocity (m/hr)	Seed sludge concentration (g VSS/L)	SLR* (kg COD/kg VSS.day)	SOLR* (kg COD/ m^3.day)	Ref+.
1 – 5	-	-	6 or 12-15	-	-	1
1 – 4	24 – 11	-	10.5	-	1 – 8.73	2
-	24	0.08	-	-	3	3
1- 3.5	7	0.25 – 0.35	7.8	0.2 – 1	1 – 12.5	4
1 – 3	16 – 8	0.05 – 0.1	-	0.1 – 0.6 (opt: 0.15 – 0.24)	1.5 – 9 (opt: 2 -3.6)	5

* Given ranges indicate the stepwise increases or decreases.

+ References: 1) Hulshoff Pol and Lettinga, 1986; 2) Teo et al., 2000; 3) Noyola and Moreno; 1994; 4) Wu et al., 1987; 5) Ghangrekar et al., 1996.

Ref: References, VSS: Volatile suspended solid, SOLR: Space organic loading rate, opt: Optimum.

In aerobic granulation, the light and dispersed sludge is washed out and relatively heavy granules are retained in SBRs. SBR cycle time, therefore, represents the frequency of solids discharged through effluent withdrawal and it is related to the HRT (Liu and Tay, 2004). If cycle time is too short, then solid washout can be observed. However, on the other hand, SBR cycle time serves as a main hydraulic selective pressure on the microbial community. Short cycle time stimulates microbial activity and production of extracellular polysaccharides and also improves cell hydrophobicity. HRT should be short enough to suppress the suspended growth (< 24 hr), but long enough for microbial growth and accumulation (> 3 hr) (Liu et al., 2003b). Similarly, as HRT, settling time applied during SBR operation also acts as a hydraulic selective pressure. Short settling time (as low as 1 min, Tay et al., 2001a) stimulates the production of extracellular polysaccharide and improves the cell surface hydrophobicity significantly.

Aerobic granulation is also affected by starvation and intermittent feeding. An SBR operation is a sequencing cycle of feeding, aeration, settling and withdrawal (and idle in some studies) periods. Aerobic cultures in the SBR are subjected to periodic fluctuations. During operation period, an important period of aerobic substrate starvation has been identified (Tay et al., 2001a). Under starvation conditions, bacteria became more hydrophobic which facilitates microbial adhesion and aggregation (Bossier and Verstraete, 1996; Tay et al., 2001a). Thus starvation plays an important role in the microbial adhesion process and leads to stronger and denser granules (Liu and Tay, 2004). It is also reported that a feast-fast feeding or pulse feeding of the SBR favored the formation of aerobic granules (Liu and Tay, 2004).

So far, DO concentration has not been considered as a significant parameter leading to aerobic granulation in SBRs. The reason was stated as the achievement of aerobic granulation under varied DO concentration from 0.7-1 mg/L (Peng et al., 1999; 2001) to values greater than 2 mg/L (Liu and Tay, 2004). However, it should be noted that DO values referred to the concentrations achieved through

the aeration periods of SBR cycles. In fact, up to aeration period and even in the first parts of filling period (due to high utilization rate), DO concentrations in the SBRs were detected as nearly zero (Morgenroth et al., 1997; Peng et al., 1999; 2001). In addition, aerobic granules were also developed in SBRs operated with cycles including anaerobic or anoxic periods of 1.5-2 hr (Zhu and Wilderer, 2003; Jang et al., 2003). Therefore, the effect of DO gradient achieved through the cyclic operation in SBRs may be a significant factor in aerobic granulation and should be investigated in detail.

The use of organic and inorganic nuclei (e.g. inert matters) often yields significantly beneficial effects on sludge granulation. Critical concentrations of divalent cations (e.g. Ca^{+2}, Mg^{+2} and Fe^{+2}) also have a stabilizing effect on granulation. The lower susceptibility to floatation of granules as compared with flocs is attributed to the presence of the hydrophilic ECP coating that prevents the attachment of gas bubbles (Teo et al., 2000). In UASB systems, granules are subjected to hydraulic shear forces and gas lifting. Therefore, microbial adhesion in UASB granulation, either microbe to microbe or microbe to support phase, may involve strong binding forces. Jiang et al. (2003) also indicated that addition of Ca^{+2} accelerated the aerobic granulation process, by binding to the negatively charged groups present on the bacterial surfaces and polysaccharides molecules and thus acts as a bridge to promote bacterial aggregation.

The substrate N/C ratio has a direct and profound effect on the elemental compositions and characteristics of aerobic granules (Liu et al., 2003c). Aerobic granules could be developed at substrate N/C ratio ranging from 5/100 to 30/100. Nitrifying populations in the aerobic granules increased with the increasing substrate N/C ratio (Liu et al., 2003c; Yang et al., 2003). The substrate N/C ratio also determines the respective ratio of cell oxygen, nitrogen and calcium to cell carbon. As substrate N/C increases, cell N/C ratio also increases while the cell oxygen to carbon ratio displays a decreasing trend. In addition, cell hydrophobicity of aerobic granules is inversely related to the ratio of cell oxygen

to cell carbon or to the N/P ratio (Rouxhet and Mozes, 1990; Liu et al., 2003c). It is stated that increased C/N ratio stimulated the production of ECPs resulting in improved bacterial attachment to the solid surfaces (Schmidt and Ahring, 1996).

The effect of pH and temperature on aerobic granulation is lacking, but suggested to be not as significant as in the anaerobic granulation process (Liu and Tay, 2004). Reactor configuration for aerobic granulation is important in improving selection of granules by the difference in settling velocity. Therefore, a high H/D ratio (reactor height/reactor diameter) is usually preferred in SBRs (Beun et al., 1999; Liu and Tay, 2004). As mentioned previously, hydrodynamic shear force plays a crucial role also in anaerobic granulation, which can be achieved in UASB reactors by liquid upflow velocity. However, development of anaerobic granules in continuously stirred tank reactors indicated that rather than the type of the reactor, the way of operation also affects the granulation process (Vanderhaegen et al., 1992). The effect of nature of flow on anaerobic granulation has not been clarified yet (Liu and Tay, 2004).

2.2.2. Granulation Mechanisms

Although, use of granules has been recognized as one of the most efficient biological processes for wastewater treatment, the mechanisms for the formation of both anaerobic and aerobic granules have not been understood because of its complexity. Bacteria would prefer a dispersed, rather than aggregated, state under normal conditions. It is most likely that microbial granulation is a response to stressful environments that make bacteria aggregate together.

Both a short hydraulic retention time and a relative high shear force are found favorable for aerobic granulation in SBRs (Beun et al., 1999). In other words, aerobic granulation is driven by selective pressure (Qin et al., 2004). The formation and characteristics of the granules may be controlled by manipulating

the selective pressure (Liu and Tay, 2004). A high shear force and selective pressure result in a significant increase in cell surface hydrophobicity, which might be the main triggering force in the initiation of aerobic granulation (Liu et al., 2003b; Liu and Tay, 2004). According to the thermodynamic theory, increasing cell surface hydrophobicity would cause a corresponding decrease in the excess Gibbs energy of the surface and promote cell-cell interaction to further drive the self-aggregation of bacteria out of suspending liquid (hydrophilic phase).

It has been generally accepted that the formation of granules is correlated with the production of ECP (Schmidt and Ahring, 1996). ECPs in granules consist of mainly protein, polysaccharides and also lipids and its composition affects the surface property and physical properties of granules. The production of ECP can change the surface charge of the bacteria, resulting in aggregation, but on the other hand, too much ECP can also cause deterioration and repulsion can occur. It has been stated that contribution of cell protein is less important than of cell polysaccharides to aerobic granulation (Tay et al., 2001b; Liu and Tay, 2002; Qin et al., 2004). Extracellular polysaccharides play also a crucial role in maintaining the structural integrity of the anaerobic granule (Schmidt and Ahring, 1996). An environmental stress such as a short settling time results in the stimulation of extracellular polysaccharide production. Bacteria can change their surface hydrophobicity as a response to certain environmental stresses such as starvation, nutrient limitation, high pH, high temperature and high shear force (Tay et al., 2001b; Liu et al., 2003b; Qin et al., 2004). When bacterial surfaces are highly hydrophobic, irreversible adhesion would occur (Qin et al., 2004).

Based on microscopic observations, a hypothesis for the granulation process was formulated (Beun et al., 1999). At the beginning, filamentous fungal pellets dominated the SBR. These pellets functioned as an immobilization matrix in which bacteria could grow out to colonies. After a certain time the fungal pellets fell apart due to lysis in the inner part of the pellets, the bacterial colonies could

then remain in the reactor because they were large enough to settle sufficiently fast, which further grew out to granules.

Since granulation is almost entirely studied in the context of methanogenic systems, it is regularly hypothesized that the specific syntrophic bacterial interactions in this process are the main cause of the anaerobic granulation. On the other hand, it can be stated that in the UASB reactor the microorganisms have to grow in a granule because otherwise they would be washed out due to continues upward liquid velocity in the UASB reactor (Beun et al., 1999). In other words, upflow velocity in the UASB reactor creates a selective pressure to which the organisms have two responses: to be washed out or to bind together and form easily settleable granules.

A number of models for anaerobic granulation have been developed over the past 20 years (Liu and Tay, 2004). The proposed models include the physico-chemical models (inert nuclei model, selection pressure model, multi-valence positive ion-bonding model, ECP bonding model, synthetic and natural polymer-bonding model, secondary minimum adhesion model, local dehydration and hydrophobic interaction model, surface tension model), structural models (Capetown's model, Spaghetti model, syntrophic microcolony model, multi-layer model) and etc. (Liu and Tay, 2002). A new theory for anaerobic granulation was also proposed by Teo et al. (2000), namely proton translocation-dehydration theory, which is based on the proton translocating activity on bacterial surfaces.

Although mechanisms and models for aerobic granulation have been described, they do not provide a complete picture of the granulation process. Based on the existing mechanisms, Liu and Tay (2002) and Liu et al. (2003b) proposed a model for granulation process consisting of four steps:

- Step 1: It is the physical movement to initiate bacterium to bacterium contact. The factors involved in this step are the hydrodynamics, diffusion mass transfer, gravity, thermodynamic effects and cell mobility.
- Step 2: This step is the stabilization of multicell contacts resulting from the initial attractive force. The initial attractive forces are the physical forces (e.g. Van der Waals forces, opposite charge attraction, thermodynamically driven reduction of the surface free energy, surface tension, hydrophobicity, filamentous bacteria that can bridge individual cells), chemical forces and biochemical forces including cell surface dehydration, cell membrane fusion, signaling and collective action in bacterial community.
- Step 3: It is the maturation of cell aggregation through production of ECP, growth of cellular cluster, metabolic change and environment-induced genetic effects that facilitate the cell-cell interaction and result in highly organized microbial structure.
- Step 4: It is the shaping of the microbial aggregate by hydrodynamic shear forces.

Despite of the mechanisms given, the events at molecular or genetic level still require a more profound understanding of the mechanisms. Intercellular communication and multicell coordination are known to contribute the organization of bacteria into spatial structures. Signal exchange among individual cells allows the entire population to choose an optimal way of interacting with the environment. Cell-cell signaling is effective in developing granules and organizing the spatial distribution of the bacteria in the granules (Liu and Tay, 2004). In other words, bacteria in a multispecies biofilm are not randomly distributed but rather organized to best meet the needs of each. They distribute themselves according to which can survive best in the particular microenvironment and also based on symbiotic relationships between the groups of bacteria (Watnick and Kolter, 2000).

2.3. Treatment of Low Strength Wastewaters

A number of industrial wastewaters in developing countries, in general from the food processing sectors, are frequently found with low strength since the water management is not always very efficient and large volumes of dilute wastewaters are produced (Kato et al., 1997). Thus, low strength wastewaters can be characterized as those dilute industrial effluents of less than 2000 mg/L COD, which may contain a variety of biodegradable compounds such as simple short chain volatile fatty acids (VFA), alcohols and carbohydrates, as well as proteins, fats and long-chain fatty acids in some cases (Lettinga and Hulshoff Pol, 1991). Examples are effluents from alcoholic and soft drink bottling industries, paper recycle and papermaking mills, fruit and vegetable canneries, and malting and brewing processes. Others define the low strength wastewaters as those with COD value less than 1000 mg/L (Mergaert et al., 1992; Ndon and Dague, 1994) such as municipal sewages.

At the present time, activated sludge processes are widely used for low strength wastewater treatment (e.g. municipal wastewater). Large-scale success of the activated sludge process does not mean that it has no problems. Achieving reliable treatment over which the operator has little control, either in flow rate or composition, presents a great challenge to engineers and operators today. In addition, successful operation depends upon a biological process in which the microorganisms themselves can be monitored and controlled with only coarse tools (Rittman and McCarty, 2001). The ecology of the system can change from day to day, leading to significant problems, such as sludge bulking, a condition under which the microbial floc does not compact well. Sludge bulking makes it difficult to capture and recycle the microorganisms fast enough to maintain the desired large biomass concentration in the reactor. It is somewhat amazing that, despite of all uncertainties and uncontrollable factors involved, the activated sludge process actually has the reliability that it does have (Rittman and McCarty,

2001). Many modifications of the basic activated sludge process have evolved since 1914 such as contact stabilization, step aeration, extended aeration etc., which were developed to overcome the problems encountered. It is sure that conventional activated sludge systems, as well as its modifications, have a significant place in municipal wastewater treatment due to high removal efficiencies achieved (90-95%, in terms of 5-day BOD) (Rittman and McCarty, 2001). However, they also have some disadvantages. Due to the adequate aeration requirement, high sludge production (0.4 g dry weight/g COD removed), the high disposal costs of the sludge and in turn the considerable investment, operation and maintenance costs, it is often questioned why aerobic treatment of low strength wastewater is not replaced more rapidly by the economically more attractive and the conceptually more holistic direct anaerobic treatment (Mergaert et al., 1992).

However, some common problems have arisen from anaerobic treatment of low strength wastewaters. The possible problems are either wastewater or reactor design related. Wastewater related problems such as low substrate concentration occurring inside the reactor, the possible presence of DO and lower temperatures, are inherent due to the characteristics of wastewater (Kato et al., 1997). Especially, low strength wastewaters pose a special problem associated with the fact that at lower substrate concentrations the diffusion rate of substrate to biomass (the rate-driving force) will be lower. The problems become more pronounced when biomass population is low, as in conventional anaerobic digestion.

The loading rates permissible in an anaerobic treatment process are primarily dictated by the sludge retention in the anaerobic reactor. As mentioned previously, the maintenance of a high solid retention time (SRT) has been the major problem in the practical application of the process, especially for wastes with a COD below about 3000 mg/L (Lettinga et al., 1980). Obviously, a waste treatment process for low strength wastes is an economical one if large volumes of waste can be forced through the system in a relatively short time period. For this purpose, processes

are required in which the biomass retention time can be controlled independently of the wastewater flow rate. Conventional anaerobic treatment processes of the flow-through type are therefore inadequate to treat low strength wastes. Therefore, an anaerobic process that can overcome these problems by fostering close proximity between biomass and substrate is a system that is able to retain high populations of granular biomass and select for microbes, which are able to grow at low substrate concentrations (Ndon and Dague, 1994). As a consequence of the high biomass concentrations, it is possible to maintain a sufficient amount of very slow growing methanogenic bacteria in the reactor. These organisms are necessary for removing the organic pollutants from the wastewater, as they fulfill the terminal COD removal step in the anaerobic digestion process (Van Der Last and Lettinga, 1992).

The solution for the biomass retention problem resulted in the development of different anaerobic reactor configurations (advanced systems) over the last decade. These new advanced (high-rate or biomass retained) anaerobic reactors such as UASB, anaerobic filter (AF), anaerobic fluidized bed (AFB), anaerobic attached film expanded bed (AAFEB) and expanded bed (EB) reactors have an excellent biomass retention and significant difference between the HRT and SRT. These systems offer great opportunities for the treatment of a large variety of medium and low strength wastewaters (Yu and Anderson, 1996). Besides, these systems might also overcome the other reactor design related problems of conventional anaerobic treatment systems such as unstable operation (Yu and Anderson, 1996).

Since 1980, a considerable amount of research has been carried out on treatment of low strength wastewaters (especially municipal wastewater) by using various anaerobic reactor types. Results of some of the researches on treatability of low strength wastewaters in varied advanced anaerobic reactors are given in Table 2.3. As seen in Table 2.3, low strength wastewater treatment in advanced anaerobic

reactors (if high biomass retention is maintained) and achievement of high COD removal efficiencies is possible.

Table 2.3. Results of the low strength wastewater treatment studies in advanced anaerobic reactors.

Reactor type*	Influent COD (mg/L)	OLR (g COD/L.day)	COD removal efficiency (%)	HRT (hr)	Reference
UASB	422-722	6.8	>95	2.6	Kato et al., 1997
EGSB	100-200	12	>80	1	
AF	288	0.32	79	24	Kobayashi et al., 1983
UASB	600	3.6-6	90	3	Gnanadipathy and Polprasert, 1993
AFBR UASB	250 (as BOD)	-	80	3	Kida et al., 1991
UASB	630	-	77	8	El-Gohary and Nasr, 1999
UASB	630	-	59	9.8	Forster and Wase, 1983
ASBR	1000	-	87	12	Ndon and Dague, 1994
	400	-	91		
ASBR	500	-	80	8	Rodrigues et al., 2003
ABR	500	-	80	10	Langenhoff and Stuckey, 2000
			60	10	

* EGSB: Expanded granular sludge bed, AF: Anaerobic filter, AFBR: Anaerobic fluidized bed reactor, ASBR: Anaerobic sequencing batch reactor, ABR: Anaerobic baffled reactor.

In view of the results of recent research work with advanced anaerobic reactors, the application of anaerobic digestion for the treatment of low strength wastewaters even at lower temperatures may be foreseen. Kida et al. (1991) compared the use of UASB reactor and AFBR and concluded that AFBR was very robust and had the best performance at low temperature (down to 15^0C) and low HRTs (3 hr) (Table 2.3). Langenhoff and Stuckey (2000) also investigated the effect of temperature on treatment of low strength wastewaters. They achieved 80% COD removal, decreasing down to 60% with the decrease of the temperature from 35 to 10^0C (Table 2.3).

Yu and Anderson (1996) indicated that among the advanced anaerobic reactors, the EB proved to be the most efficient with excellent effluent quality with a very short HRT. A slightly lower efficiency results from with the use of UASBs and AFs. So far, few full-scale AFB/EB systems have been commissioned because of high energy demand for fluidization and the difficulty in construction and operation. The UASB design has been considered to be the most attractive reactor system due to its simplicity, low investment and operation costs as well as long experience in the treatment of a wide range of industrial wastewaters. Thus, the UASB system is the most common full-scale anaerobic treatment system for municipal wastewater.

The study of Kato et al. (1997) that is given in Table 2.3 has a significance related with the aim of the thesis. In addition to the investigation of low strength wastewater in UASB and EGSB reactors, Kato et al. (1997) also evaluated the DO effect on the methanogenic activity of granular sludges and thus on treatment performance. It was found that the methanogens in the granular sludge had a high tolerance to oxygen, which was highly dependent on the substrate. Absence of substrate during the exposure period drastically decreased the oxygen tolerance; however, some oxygen tolerance was still evident. Results also revealed that DO in the aerated feed solution (3.8 mg O_2/L influent) was rapidly consumed in both

of the UASB and EGSB reactors and did not affect the treatability of low strength wastewaters.

Recently, treatment of low strength wastewater (synthetic wastewater) has been studied with aerobic granules developed in laboratory-scale SBRs (Peng et al., 1999, 2001; Yang et al., 2003; Jang et al., 2003; Hu et al., 2005b). Peng et al. (2001) studied the carbon and nitrogen removal at low DO concentrations (0.8 mg/L). High COD and NH_3-N removal efficiencies (95 and 99%, respectively) were obtained while total nitrogen removal was reported as 75% at an influent COD and NH_4Cl of 600 and 250 mg/L, respectively. Experimental results indicated that low DO conditions did not negatively affect the treatment efficiencies of aerobic granules developed in SBRs (Peng et al., 1999, 2001; Hu et al., 2005b). Aerobic granules developed under alternating aerobic and anoxic conditions displayed high COD removal efficiencies up to 95% for the treatment of synthetic wastewater with COD concentration of 500-600 mg/L (Jang et al., 2003). It was observed that both nitrification and denitrification occurred in the SBR, with increased nitrification efficiency up to 97%.

The use of aerobic granules might be considered an alternative for the treatment of low strength wastewaters. Aerobic granules developed under cyclic aerobic / anoxic (or anaerobic) and thus might contain nitrifying and denitrifying bacteria might even be used for the total nitrogen removal. However, the use of aerobic granules does also have some drawbacks. Aerobic granules were developed mostly in SBRs which are operated in discontinuous mode. Therefore, treatment of low strength wastewaters, which are usually produced in large amounts, may not be feasible in SBRs. In addition, despite of many advantages the application of aerobic granules for treatment purposes is still questionable due to the drawbacks such as low stability of aerobic granules compared to anaerobic granules (Liu and Tay, 2004). Aerobic granules are not stable as anaerobic granules due to their high-grow rates (Liu and Tay, 2004).

The feasibility of low strength wastewater treatment with anaerobic granular cultures using aerated feed (Kato et al., 1997) and with aerobic granules at low DO encouraged this study. Therefore, use of combined cultures constituted of anaerobic and aerobic cultures for the treatment of low strength wastewaters in one reactor under alternating anaerobic / aerobic and/or microaerobic conditions might be proposed as an alternative to the conventional activated sludge systems due to the following factors: the alternating conditions might result in less amount of air requirement. Besides, due to alternating anaerobic / aerobic and/or microaerobic conditions, low DO conditions will occur in the system periodically. Oxygen transfer from air to liquid at low DO is more rapid than that of high DO; less oxygen is required, which means energy saving and a lower operating cost (Peng et al., 2001). Due to the periodic low DO conditions and co-existence of slow-growing anaerobic cultures sludge production might be lower compared to conventional activated sludge systems. Thus, the sludge amount produced in conventional activated sludge systems and in turn its handling, transport and disposal problems may be overcome with the combined anaerobic and aerobic cultures.

The alternating conditions and co-existence of both anaerobic and aerobic cultures in same reactor might also encourage the complete nutrient removal (both nitrogen and phosphorus). The combined cultures might overcome the drawbacks of aerobic granulation technology such as stability due to co-existence of slow-growing anaerobic cultures (Liu and Tay, 2004; Liu et al., 2004). In addition, the use of combined cultures might become more attractive due to further advantages such as methane production i.e. renewable energy generation by anaerobes constituting the combined cultures. As a result, due to the possible advantages and ability to overcome the drawbacks of conventional activated sludge systems, it will be worthwhile to investigate the treatment of low strength wastewater with combined anaerobic and aerobic cultures.

CHAPTER 3

MATERIALS AND METHODS

3.1. Seed Cultures

Mixed anaerobic digester sludge and aerobic activated sludge were used as the seed cultures in the experiments. Mixed anaerobic digester sludge and aerobic activated sludge were obtained from anaerobic sludge digesters and the recycle line of the activated sludge units of the Greater Municipality of Ankara Central Wastewater Treatment Plant, respectively. Anaerobic granular cultures that were also used in the experiments were obtained from the UASB reactors of the Wastewater Treatment Plant of Tekel Pasabahce Liquor Factory located in Istanbul.

3.2. Basal Medium and Original Municipal Wastewater

Basal medium (BM) containing all the necessary micro- and macro-nutrients were used in the experiments. The BM constituents (Merck, Germany) and concentrations of each (given in parentheses as mg/L) are as follows: NH_4Cl (400), KCl (400), $(NH_4)_2HPO_4$ (80), $CaCl_2$ (80), $CoCl_2.6H_20$ (10), $Na_2PO_4.12H_2O$ (10), KI (10), $CuCl_2.2H_20$ (0.5), $ZnCl_2$ (0.5), $AlCl_3.6H_20$ (0.5), $Na_2MoO_4.2H_2O$ (0.5), H_3BO_3 (0.5), $NiCl_2.6H_20$ (0.5), $Na_2WO_4.2H_2O$ (0.5), $Na_2SeO_3.5H_2O$ (0.76), cysteine (10), $NaHCO_3$ (6000), yeast extract (50) and resazurin (provided by Dr. Daniel H. Zitomer, Marquette University, Milwaukee, Wisconsin) (1.04) as an oxidation-reduction indicator dye (Demirer and Speece, 1998; Ferguson, 1999).

The original municipal wastewater was obtained from the effluents of primary settling tanks of the Greater Municipality of Ankara Central Wastewater Treatment Plant. The characterization of the municipal wastewater is given in Appendix A.

3.3. Experimental Procedure

The experiments were conducted in two parts, namely, semi-continuous reactor and continuous reactor experiments. Continuous reactor experiments were performed in Upflow Sludge Blanket (USB) reactors, therefore, named as "USB reactor experiments" from this part forward. The followed experimental steps and the main aim of each part are briefly given in Table 3.1.

Table 3.1. The experimental steps followed and the main objectives of each step.

Main objectives / Experimental steps	A) Development of combined anaerobic and aerobic cultures B) Granulation C) Low strength wastewater treatment
1. Semi-continuous reactor experiments	
Studies 1, 2, 3, 4	A, B
2. USB reactor experiments	
Study 1	A, B
Study 2	A, B, C
Study 3	A, C

3.3.1. Semi-Continuous Reactor Experiments

In semi-continuous reactor experiments, it was aimed to develop combined anaerobic and aerobic cultures in granular form from a mixture of suspended anaerobic and anaerobic cultures under alternating aerobic and/or microaerobic / anaerobic conditions. First three semi-continuous reactor studies, namely study 1, 2 and 3, were operated to investigate the possibility of granulation under the mentioned conditions and factors affecting the granulation. The results of these studies were used in the set-up of semi-continuous reactor study 4, where the physical (settling velocity, particle size) and microbial (SMA and SOUR) characteristics of the developed combined cultures / granules were determined. In all semi-continuous reactor experiment studies, similar set-up and experimental procedures were followed. The differences among these set-ups were described in the following sections (Sections 3.3.1.1, 3.3.1.2, 3.3.1.3 and 3.3.1.4).

Semi-continuous reactor experiments were performed in 110 mL reactors with 50 mL effective volume. The reactors were seeded with 40 mL of mixed anaerobic digester sludge and aerobic activated sludge (50:50 v/v), which were previously screened through a 1 mm sieve to remove the debris and fibers. Ten mL of concentrated BM was added to the reactors to achieve the concentrations of the BM constituents given in Section 3.2. The reactors were then flushed with N_2/CO_2 (70/30) gas mixture for 3-4 minutes (to maintain anaerobic conditions) before sealing with rubber septa. To maintain alternating cyclic aerobic and/or microaerobic / anaerobic conditions, the reactors were maintained on a continual two-day wasting / feeding schedule (Figure 3.1). This schedule is a modified form of the procedure applied by Ferguson (1999).

At the start of the schedule (Day 1) as well as on all odd days, the excess headspace gas was released using a 100 mL glass gas-tight syringe and volume was recorded. Additionally, 10 mL of mixed liquor was wasted (providing a solid retention time (SRT) of 10 days) and 10 mL of BM was added with glass gas-tight

syringe. Sludge wasting and subsequent BM addition were only done once in two days (on the first day of the schedule, Figure 3.1). Primary substrate was daily fed to the reactors achieving COD loading rate of 500 mg/L.day (or 25 mg COD/day). The COD equivalences and the main properties of the primary substrates used in the experiments are given in Appendix B. At the start of Day 2 (even days, Figure 3.1), after measuring and releasing the headspace gas, pure oxygen (99.99%, Oksan Ltd.) at ambient temperature was injected into the headspace of the reactors using syringe fitted with pressure-locked valve.

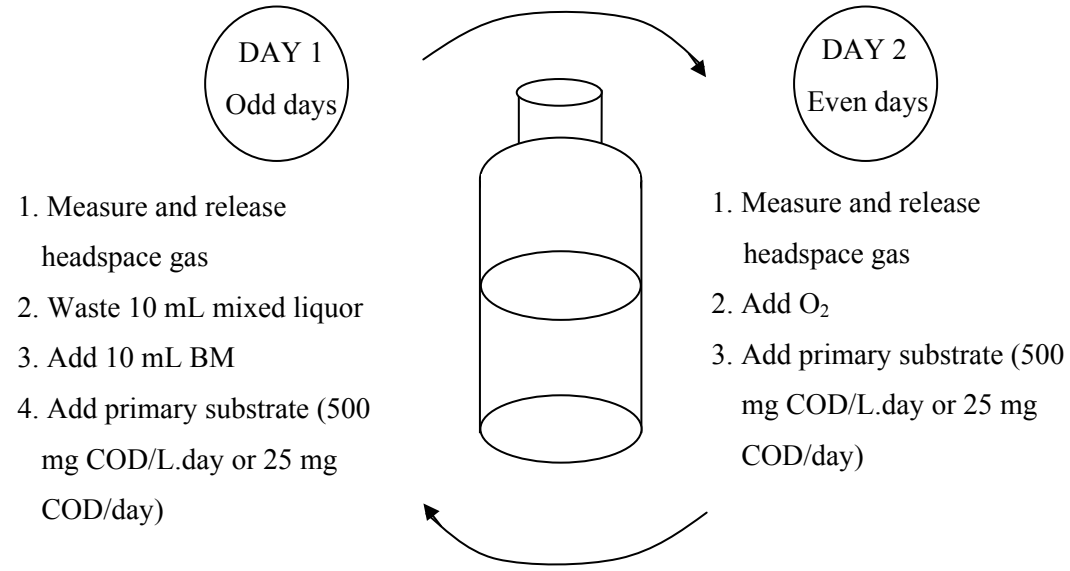


Figure 3.1. Schematic diagram of the continual two-day schedule (wasting/feeding cycle).

Strictly anaerobic (no oxygen and seeded with only anaerobic digester sludge) and aerobic control (maximum oxygen dose and seeded with only aerobic activated sludge) reactors were also set-up. The reactors except the controls were referred as test reactors from this part forward. All the reactors were visually

monitored for the changes in their contents (color of the supernatants and cultures) and in the sizes of the granules with respect to time.

3.3.1.1. Semi-Continuous Reactor Study 1: Effect of Oxygen Dose

The first study was conducted to determine the effect of oxygen dose on granulation of mixed suspended anaerobic and aerobic seed cultures. The test reactors had initial mixed liquor suspended solid (MLSS) and mixed liquor volatile suspended solid (MLVSS) concentrations of 8636 ± 300 and 4588 ± 57 mg/L, respectively. The initial anaerobic to aerobic MLVSS ratio by weight was 2.5. All the reactors were incubated and maintained on continual two-day schedule (Figure 3.1) on a shaker table at 35 ± 1 ^{0}C in the dark at 160 rpm. In order to observe the effect of oxygen dose on the cultures and granulation, five oxygen doses were studied. Oxygen doses were based on the percent of the total COD added (50 mg COD) ranging from 10 to 120% (Appendix C). Oxygen volumes injected to the test reactors on Day 2 (even days, Figure 3.1) were 4, 12, 23, 38 and 45 mL (at standard temperature and pressure) corresponding to the 10, 30, 60, 100 and 120% of the total COD added, respectively (Appendix C). To investigate the substrate type effect, two primary substrates were used, namely ethanol and glucose+acetic acid (HAc) (1:1 as COD). Each of the oxygen doses was studied for both of the substrates. All the test reactors (fed with varied oxygen doses for each substrate) and the control reactors (for each substrate) were set-up in duplicates.

Glucose was selected as one of the substrate types because it is stated in literature that the presence of high-energy substrates (e.g. glucose, sucrose) and consequently higher concentrations of acidogens are essential for a well-granulated biomass (Teo et al., 2000). Many types of anaerobic bacteria are involved in the biodegradation process, thus they must live in a close synergistic relationship where products, such as H_2 and other intermediates, can be efficiently

transferred among the respective bacterial groups (Thiele and Zeikus, 1988; Liu et al., 2003a). The stated requisites would eventually lead to the formation of microcolonies or consortia, i.e. initial granules, which is suggested in syntrophic microcolony model (Beun et al., 1999; Liu et al., 2003a). Thus, HAc was applied with glucose to support the growth of acetogens and in turn to make the bacterial interactions among the respective bacterial groups (acidogens and acetogens) possible. The reason of selecting ethanol as the other substrate type was the achievement of pelletized cultures in the study of Ferguson (1999) where lactic acid was applied during the continual two-day wasting/feeding cycle. Ethanol and lactic acid are found at the same tropic level of anaerobic degradation pathway. Therefore, because the microorganism types involved in lactic acid degradation would be similar to the ones involving in ethanol degradation, it might be possible to observe pelletized cultures or granules if ethanol is used as the primary substrate.

3.3.1.2. Semi-Continuous Reactor Study 2: Effect of Substrate Type, Shaking and Sludge Wasting

Three sets of semi-continuous reactors (Sets A, B and C) were set-up. Test reactors seeded with a mixture of suspended anaerobic and aerobic cultures had initial MLSS and MLVSS concentrations of 16990 ± 247 and 7842 ± 106 mg/L, respectively. The initial anaerobic to aerobic MLVSS ratio by weight was 6.6.

The results of the first semi-continuous reactor study (Section 4.1.1) revealed that granulation was possible with both of the primary substrates (ethanol or glucose+HAc). In order to determine the separate effects of glucose and HAc on granulation four carbon sources, namely, ethanol, glucose, HAc, and glucose+HAc were used as primary substrate in each set. For each substrate, two oxygen doses, namely, 60 and 120% of the total COD added (corresponding to 23 and 45 mL, respectively) were studied. All the test reactors (fed with varied

oxygen doses for each substrate) and the control reactors (for each substrate) of each set were set-up in duplicates.

Each set was maintained on continual two-day schedule (Figure 3.1) with the differences given in Table 3.2. As seen in Table 3.2, sludge wasting and BM addition were not applied to Sets A and B, but to Set C. Besides, the reactors in Set A were incubated at 35 ± 1 ^{0}C on a shaker table in the dark at 160 rpm, while the others in Sets B and C were incubated in a temperature-controlled room at 35 ± 2^{0}C (without shaking). The aim of conducting these three sets is briefly tabulated in Table 3.3 by means of indicating the main differences among the sets. As seen in Tables 3.2 and 3.3, the effect of shaking on granulation process would be determined by comparing the results of Sets A and B. Similarly, the effect of sludge wasting on granulation would be determined by comparing the results of Sets B and C.

Table 3.2. Continual two-day schedule of each set operated in study 2.

	SET A	SET B	SET C
DAY 1 (odd days)	* Measure and release headspace gas *Add primary substrate	* Measure and release headspace gas *Add primary substrate	* Measure and release headspace gas * Waste 10 mL mixed liquor * Add 10 mL BM * Add primary substrate
DAY 2 (even days)	* Measure and release headspace gas * Add O_2 * Add primary substrate	* Measure and release headspace gas * Add O_2 * Add primary substrate	* Measure and release headspace gas * Add O_2 * Add primary substrate

Table 3.3. The aim of conducting three sets and the main differences among them.

	SET A	SET B	SET C
Shaking	+	_	_
Wasting mixed liquor +adding BM	_	_	+
Comparison of Sets A and B	Effect of shaking		_
Comparison of Sets B and C	_	Effect of wasting	

3.3.1.3. Semi-Continuous Reactor Study 3: Effect of Initial Anaerobic to Aerobic Biomass Ratio and Total Biomass Concentration

The third study was designed to investigate the effect of initial ratio (R) of anaerobic to aerobic biomass (as MLVSS) and the initial total biomass concentration on granulation. Test reactors were seeded with mixed anaerobic digester sludge and aerobic activated sludge in required amounts to achieve initial R-values (anaerobic to aerobic MLVSS ratio) as 1.7, 2.9, 4.6, 5.7 and 7.5. The initial average total MLSS and MLVSS concentrations in the test reactors with different R-values were set approximately at 7800 and 3200 mg/L, respectively. The studied R-values and MLSS and MLVSS concentrations in the test reactors corresponding to each R are given in Table 3.4.

To investigate the effect of initial total biomass concentration on granulation the anaerobic and aerobic seed sludge concentrations were doubled for the two of the R-values, namely, 4.6 and 5.7 (Table 3.4). For each anaerobic and aerobic sludge concentration achieved in the test reactors, strictly anaerobic and aerobic control reactors were also set-up. All control and test reactors were studied in duplicates. The reactors were maintained on continual two-day schedule (Figure 3.1), but without wasting of the mixed liquor and adding BM on the first day of the schedule (odd days). As primary substrate and oxygen dose, ethanol and 45 mL of

pure oxygen (120% of the total COD added) were fed to the reactors. All the reactors were incubated at 35 ± 1 ^{0}C on a shaker table in the dark at 160 rpm.

Table 3.4. Studied R-values and MLSS and MLVSS concentrations in the test reactors.

R-values studied	Initial sludge concentrations (mg/L)					
	Anaerobic		Aerobic		Total	
	MLSS	MLVSS	MLSS	MLVSS	MLSS	MLVSS
1.7	5213 ± 23	1973 ± 10	1613 ± 21	1151 ± 32	6826	3123
2.9	6226 ± 15	2356 ± 11	1152 ± 12	822 ± 24	7378	3178
4.6	6950 ± 64	2630 ± 26	806 ± 13	575 ± 14	7757	3206
4.6*	13901 ± 106	5261 ± 49	1613 ± 15	1151 ± 15	15514	6411
5.7	7240 ± 85	2740 ± 22	672 ± 6	479 ± 9	7912	3219
5.7*	14480 ± 203	5480 ± 45	1344 ± 12	959 ± 41	15824	6439
7.5	7530 ± 25	2850 ± 30	531 ± 10	379 ± 14	8061	3229

* Anaerobic and aerobic seed sludge concentrations were doubled.

3.3.1.4. Semi-Continuous Reactor Study 4: Determination of Physical and Microbial Characteristics

A set of semi-continuous reactors was conducted to examine the physical (settling velocity, particle size) and microbial (SOUR and SMA) characteristics of the granules developed from a mixture of suspended anaerobic and aerobic cultures. The reactors were maintained on continual two-day schedule (Figure 3.2), which was modified and got the final form based on the results of previous three semi-continuous reactor studies.

The reactors seeded with mixed anaerobic digester sludge and aerobic activated sludge (50:50 v/v) had MLSS and MLVSS concentrations of 18640 ± 104 and 7609 ± 122 mg/L, respectively. The initial ratio of anaerobic to aerobic MLVSS by weight was 4.13. As primary substrate ethanol was fed to the reactors daily (500 mg/L.day or 25 mg COD/day). Two oxygen doses, namely, 60 and 120% of the total COD added were applied (oxygen volumes of 23 and 45 mL, respectively). Strictly anaerobic and aerobic control reactors were also set-up. All the reactors were incubated at 35 ± 1 ^{0}C on a shaker table in the dark at 160 rpm.

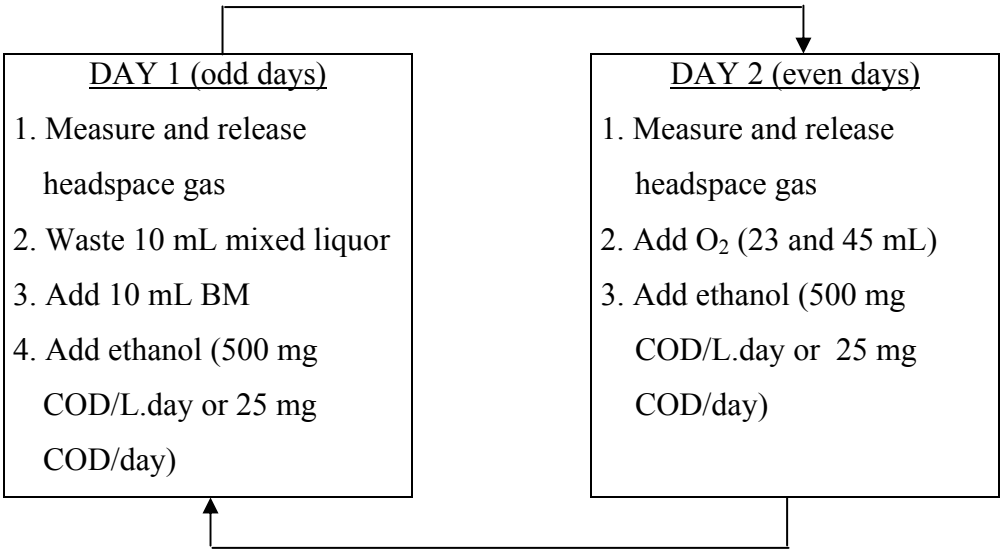


Figure 3.2. Schematic diagram of continual two-day schedule applied in study 4.

For each of the four types of cultures (i.e. anaerobic control, aerobic control, 60%-oxygen fed test and 120%-oxygen fed test cultures), six parallel reactors were conducted. During the operation period, one of the six parallel reactors of each type was removed periodically for image analyses (IA), determination of the particle sizes, SMA and SOUR activities of the developed granules. In other words, time dependent development of granules and the changes in their activities were examined. Settling velocity test was performed for the granules developed in

the last of the six parallel reactors that were removed at the end of the operation period. In addition, each removed reactor was analyzed for its DO, effluent soluble COD (SCOD) and MLVSS contents. To detect the conditions prevailing in the reactors (i.e. anaerobic, aerobic or microaerobic), their contents were initially analyzed for DO before being used for the other analyses.

3.3.2. USB Reactor Experiments

These experiments were performed to develop combined cultures / granules from a mixture of anaerobic and aerobic cultures in continuously operated USB reactors under alternating aerobic and/or microaerobic / anaerobic conditions. It was further aimed to treat low strength wastewaters in the USB reactors with the combined anaerobic and aerobic cultures / granules developed.

It was initially aimed to investigate the possibility of granulation of a mixture of suspended anaerobic and aerobic cultures in USB reactors. Therefore, first USB reactor study (study 1) was conducted. Based on the results of the first USB reactor study, two other USB reactor studies (studies 2 and 3) were carried out.

3.3.2.1. Study 1: Development of Combined Granules from Suspended Anaerobic and Aerobic Cultures

The first USB reactor study was performed to investigate the granulation possibility of a mixture of suspended anaerobic and aerobic cultures in USB reactors, to determine the aeration rate leading to alternating aerobic and/or microaerobic / anaerobic conditions in the reactors and to examine the effects of aeration strength on granulation.

Experiments were performed in two identical USB reactors constructed of cylindrical glass columns (Figure 3.3). Each reactor had an internal diameter of 4.4 cm, height of 67 cm and an active volume (liquid volume) of 0.79 L. The reactors were continuously aerated via porous air stones located at the bottom of the reactor. Two aeration flow rates, namely 10 and 100 mL/min, were studied in order to investigate the effects of aeration strength and accordingly the DO concentration on granulation. Aeration was accomplished by air pumps (EP-9000, Rambo).

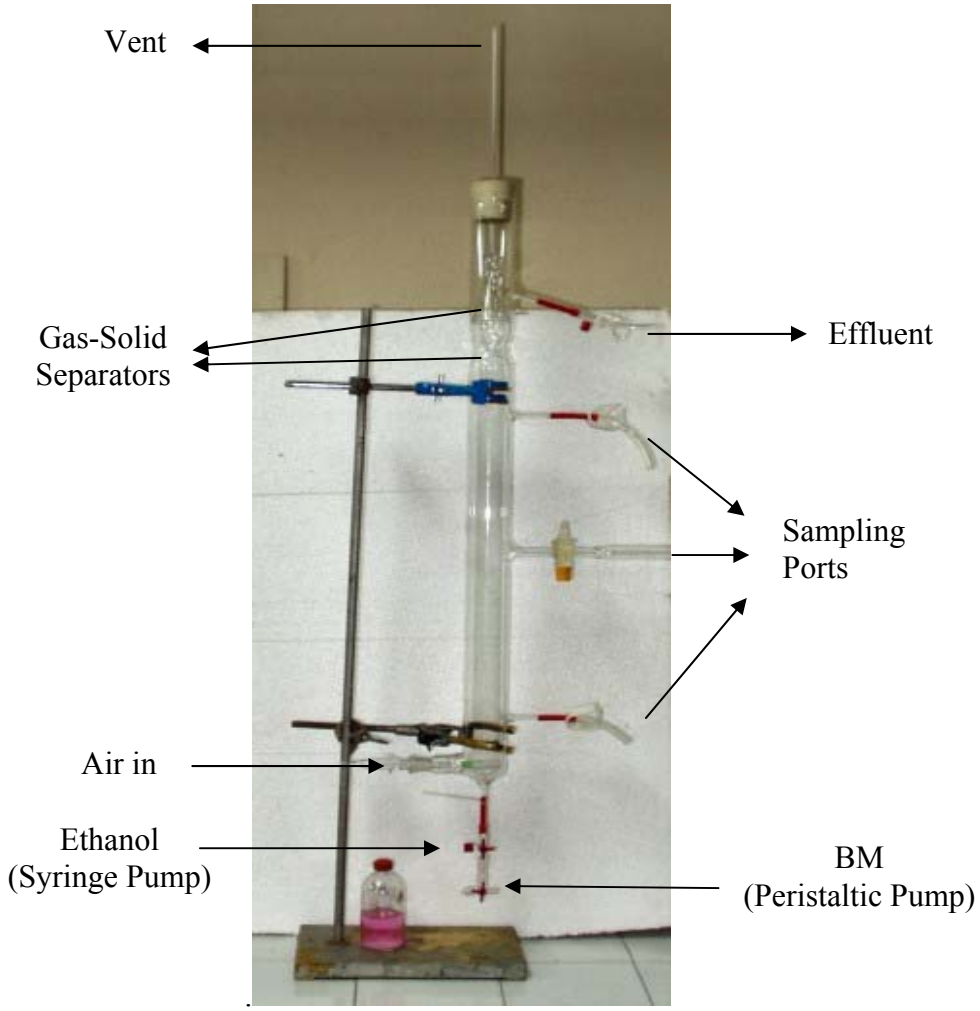


Figure 3.3. USB reactor.

Each reactor was seeded with a mixture of suspended anaerobic digester sludge and aerobic activated sludge (50:50 v/v). The amount of the seed sludge mixture, which is an important start-up parameter of the upflow-like reactors, was 8.74 ± 0.21 g/L VSS (Yu et al., 2001). The seed sludge mixture was previously screened through a 1 mm sieve to remove the debris and fibers before seeding.

Ethanol, which led to the development of the largest and the most stable granules from a mixture of suspended anaerobic and aerobic cultures in the first and second semi-continuous reactor studies (Sections 4.1.1 and 4.1.2), was used as carbon source. Ethanol and BM were continuously fed to the reactors by syringe pump (sp200i, P-74900-10, Cole-Parmer, USA) and peristaltic pump (MasterFlex L/S Standard Drive Pump, P-07521, Cole-Parmer, USA), respectively. In order to distribute the influent uniformly, glass beads (6 mm diameter) were placed at the inlet of the reactors.

The operational conditions of the reactors were assessed by the parameters such as the influent SCOD concentration, space organic loading rate (SOLR, g COD daily loaded per reactor liquid volume) and hydraulic retention time (HRT). These operating parameters and their values were determined in view of the anaerobic and aerobic granulation studies in UASB reactors and SBRs, respectively (Lettinga and Hulshoff Pol, 1986; Wu et al., 1987; Ghangrekar et al., 1996; Morgenroth et al., 1997; Beun et al., 1999; Teo et al., 2000; Yu et al., 2001). The initial influent SCOD concentration was set to be between 2-3 g/L. This value was selected considering the minimum and maximum COD levels recommended for a successful USB start-up as 1 g/L and 5 g/L, respectively (Hulshoff Pol and Lettinga, 1986). The initial SOLR was kept around 1.5 g COD/L.day (Lettinga et al., 1984), while the HRT was 1.5 day (Yu et al., 2001). The SOLR was increased in a stepwise manner by increasing the strength of the feed solution up to 3-4 g SCOD/L and/or decreasing the HRT down to 1 day, after at least 80% SCOD

reduction was achieved. The reactors were operated at 35 ± 2 ^{0}C in a temperature-controlled room.

During the start-up period, in order to investigate the changes of the sludge characteristics or granule development, sludge was periodically sampled from the ports located at different heights along the reactor (Figure 3.3). The sludge samples were analyzed for their particle size diameter, suspended solid (SS) and volatile suspended solid (VSS) contents. Performances of the USB reactors were assessed by determining the feed rate and effluent pH on a daily basis, and DO concentration in the reactors, volatile fatty acids (VFA), alkalinity, COD, SS and VSS of the effluents on a weekly basis.

3.3.2.2. Study 2: Development of Granular Combined Cultures from Suspended Anaerobic and Aerobic Cultures

The results of the first USB reactor study (Section 4.2.1) revealed that it might be possible to obtain granules from suspended anaerobic and aerobic cultures if the excessive sludge washout was prevented. As a solution to the sludge washout and combined culture development, two alternatives were proposed: 1) application of a low aeration flow rate (i.e. 10 mL/min) in periods, 2) the substitution of suspended anaerobic seed cultures with anaerobic granular sludge. Therefore, based on the seed sludge used, two USB reactor studies, study 2 and study 3, were carried out respectively for; 1) the development of granular combined cultures from suspended anaerobic and aerobic cultures, 2) the development of combined cultures from granular anaerobic and suspended aerobic cultures. The developed combined cultures / granules were further used to treat low strength wastewater.

In USB reactor study 2, it was aimed to develop combined cultures from a mixture of suspended anaerobic and aerobic cultures in granular form under alternating aerobic and/or microaerobic / anaerobic conditions. It was further

planned to treat low strength wastewaters with the combined cultures developed. Experiments were performed in one USB reactor constructed of cylindrical glass column with an internal diameter, height and an active volume (liquid volume) of 4.8 cm, 78 cm and 0.93 L, respectively (same structure as in Figure 3.3). The reactor was seeded with a mixture of suspended anaerobic digester sludge and aerobic activated sludge (50:50 v/v), which was previously screened through a 1 mm sieve. The amount of the seed sludge mixture was 3.80 ± 0.36 g/L VSS.

The reactor was aerated via a porous air stone located at the bottom of the reactor where glass beads of 6 mm diameter were placed at the inlet for uniform influent distribution. The results of the first USB reactor study (Section 4.2.1) revealed that the reactor should be aerated in periods in order to prevent sludge washout due to aeration. Therefore, among the studied aeration flow rates (10, 60 and 100 mL/min), the minimum flow rate (10 mL/min) was selected and applied 4 hours in a day. Such a periodic aeration might also supply the acclimation of anaerobic and aerobic seed cultures and in turn the enrichment of combined cultures by means of achieving periodic anaerobic and aerobic and/or microaerobic conditions in the system. The operational conditions of the reactor were evaluated by the parameters such as influent SCOD concentration, SOLR, sludge loading rate (SLR) and HRT.

The reactor was operated in three phases. Phase 1 was the start-up period for granulation. Therefore, the start-up operating parameters and loading strategy applied in Phase 1 were the same as those applied in the first USB reactor experiments (Section 3.3.2.1). Ethanol was used as carbon source. Ethanol and BM were continuously fed to the reactor by syringe pump (sp200i, P-74900-10, Cole-Parmer, USA) and peristaltic pump (High-rate pump, P-7567-70, Cole-Parmer, USA), respectively. The initial influent COD concentration (as ethanol) was set to 2.5 g/L. The initial SLR was kept around 0.2 g COD/g VSS.day (Lettinga et al., 1984), while the HRT was 1.5 day (Yu et al., 2001). The SLR was increased in a stepwise manner by increasing the strength of the feed solution up

to 3.5 g COD/L and/or decreasing the HRT down to 0.75 day, after at least 80% SCOD reduction was achieved.

After Phase 1, low strength wastewater treatment part including Phases 2 and 3 was started. In Phase 2 for adaptation of the cultures to the low strength wastewater, the strength of the synthetic wastewater (prepared with ethanol and BM as in Phase 1) was gradually decreased from 3.5 g/L to 0.57 g/L SCOD. In addition, HRT was decreased from 0.75 days to 0.33 days. In Phase 3, (resembling the low strength wastewaters) municipal wastewater was fed to the reactor.

The reactor was operated at 35 ± 2^0C in the temperature-controlled room. During the operation period, to investigate the changes of the sludge characteristics or granule development, sludge was periodically sampled for IA and determination of particle size diameter, SS and VSS contents. In order to examine the anaerobic (aceticlastic methanogenic) and aerobic microbial activity, SMA and SOUR assays were performed for the sludge samples. Performance of the USB reactor was assessed by the determination of the flow rate on a daily basis and DO concentration in the reactor, pH, VFA, alkalinity, SCOD, SS and VSS of the effluent on a weekly basis. During the municipal wastewater treatment part (Phase 3), the reactor performance was also assessed by 5-day BOD (BOD_5) concentrations. At the end of the operational period, the reactor content was analyzed for settling velocity and sludge volume index (SVI) tests.

3.3.2.3. Study 3: Development of Combined Cultures from Granular Anaerobic and Suspended Aerobic Cultures

Third USB reactor study was carried out to develop combined cultures from a mixture of granular anaerobic and suspended aerobic cultures in USB reactors for

the treatment of low strength wastewaters under alternating aerobic and/or microaerobic / anaerobic conditions.

Experiments were performed in four identical USB reactors constructed of cylindrical glass columns (Figure 3.3). Each reactor had an internal diameter of 4.4 cm, height of 67 cm and an active volume of 0.79 L. Three of the reactors (test reactors; R2, R3 and R4) were seeded with a mixture of granular anaerobic sludge and suspended aerobic activated sludge (40:60 v/v) achieving VSS concentration of 11.08 ± 0.79 g/L in each reactor. In order to be used as anaerobic control, one reactor (R1) was only seeded with anaerobic granular sludge. The amount of the granular sludge in the anaerobic control reactor was the same as the granular sludge amount seeded to the test reactors, leading to a VSS concentration of 4.15 ± 0.32 g/L.

It was also aimed in this part to investigate the effect of aeration period and accordingly the DO concentration on combined culture development and low strength wastewater treatment. Therefore, all the test reactors were aerated with a flow rate of 10 mL/min but under different aeration periods. One of the test reactors (R2) was aerated 4 hours/day, while the other one (R3) was continuously aerated one day followed by no-aeration period for the subsequent day. The remaining test reactor (R4) was continuously aerated throughout the operation period. The aeration was applied via porous air stones located at the bottom of the reactors. Anaerobic control reactor was not aerated. The reactors were operated in the temperature-controlled room at 35 ± 2^0C.

The reactors were operated in two phases (Phases 1 and 2). In both phases, low strength wastewater treatment was studied. However, synthetic wastewater was applied in Phase 1, while original municipal wastewater was used in Phase 2. In Phase 1, as carbon source glucose+HAc (1:1 in terms of COD) dissolved in BM solution was continuously fed to all reactors by a peristaltic pump (MasterFlex L/S Standard Drive Pump, P-07521, Cole-Parmer, USA). In order to distribute the

influent uniformly, glass beads (6 mm diameter) were placed at the inlet of the reactors. Low strength wastewaters are characterized as those dilute industrial effluents with COD values less than 2000 mg/L COD (Lettinga and Hulshoff Pol, 1991) and as those municipal sewages with COD values less than 1000 mg/L (Mergaert et al., 1992; Ndon and Dague, 1994). Therefore, in order to resemble low strength wastewater characteristics, the COD value of the synthetic wastewater was initially set to 1800-2000 mg/L. The strength of the wastewater was gradually decreased down to 550-600 mg COD /L, after at least 80% SCOD reduction was achieved. The HRT of the reactors was initially set as 1.5 days. In Phase 2, to investigate the effect of original wastewater on treatment performance of the reactors, municipal wastewater was fed till the end of the experiment.

The sludge samples periodically withdrawn from all reactors were subjected to SMA and SOUR assays to investigate the combined culture development. Performances of the reactors were assessed by determining the flow rate on a daily basis and DO concentration in the reactors, pH, VFA, alkalinity, SCOD, BOD_5 (during Phase 2), SS and VSS of the effluents on a weekly basis. At the end of the operational period, the reactor contents were analyzed for settling velocity and SVI tests.

3.4. Analytical Methods

3.4.1. Microbial Activity and Physical Characterization

Microbial Activity: SOUR (mg DO/g VSS.hr) assay was performed for the sludge samples withdrawn from semi-continuous reactors (study 4) and USB reactors (studies 2 and 3) in order to indicate the viable aerobic microorganisms and monitor their microbial activities. SOUR was measured following the standard methods (2710 B) (APHA, 1998) by a DO meter (9071 Model, Jenway Ltd., UK).

SMA experiments were conducted in order to determine/monitor the aceticlastic methanogenic activities of the sludge samples of semi-continuous reactors (study 4) and USB reactors (studies 2 and 3) and consequently to indicate the presence of viable methanogens. Reactors of 110 mL were seeded with the sludge to be tested and BM was added (50 mL effective volume). After flushing with N_2/CO_2 (70/30) gas mixture for 3-4 minutes (to maintain anaerobic conditions), reactors were sealed with rubber septa. Certain amount of HAc was fed to the reactors to achieve COD concentration of 3000 mg/L. Reactors were incubated in the temperature-controlled room at 35 ± 2^0C. Cumulative gas production was daily measured over 5 to 7 days and biogas methane (CH_4) content was determined. At the end of the assay defined with the cessation of the gas production, VSS contents of the reactors were measured. SMA was calculated as the initial, that is, maximum CH_4 produced per gram of VSS per hour (mL CH_4/g VSS.hr) (Zitomer and Shrout, 1998). The aceticlastic methanogenic activities of the cultures were assessed by comparing the SMA of the culture of interest to the SMA of strictly anaerobic culture.

Physical Characterization: Settling velocity tests (Etterer and Wilderer, 2001) were accomplished in a plexiglas cylinder (6 cm in diameter and 90 cm in height). Single granules were placed in the cylinder filled with water. Settling time was taken for the distance of 50 cm after the upper 30 cm of the water column where granules could reach their final settling velocity. SVI was measured according to Standard Methods (APHA, 1998).

In order to investigate the granulation process, particle size analyses were performed for the granular combined cultures developed in the USB reactor studies 1 and 2 (Sections 3.3.2.1 and 3.3.2.2) and semi-continuous reactor study 4 (Section 3.3.1.4). Particle sizes were measured by placing at least 40-50 granules / particles on a slide in a light microscope (Ernst Leitz Wetzlar, Germany) and measuring by stage micrometers (Yan and Tay, 1997). Median diameter was used

to represent the sizes of the granules (Yan and Tay, 1997). Images of the particles / granules were taken via a light microscope (Prior Laboratory Microscope Model B 3000) connected online to a computer by Pro Series, high performance CCD camera. Images were monitored by analytical imaging software, namely Image ProPlus 3.0, for the particles with sizes up to 1 mm due to the inapplicability of the analyses for larger size particles.

Throughout the semi-continuous reactor studies 1, 2 and 3, the optimum conditions (such as substrate type and oxygen dose) required for granulation and the factors affecting the granulation were determined by comparing the granulation process or granules obtained in the reactors in terms of size and durability of the granules, and the time of granulation. Because the continual two-day schedule was maintained, the reactors could not be opened for granule sampling and in turn for the microscopic analysis during the experiments in order not to disturb the headspace gas content. Therefore, during the experimental run, sludge phases in the reactors or granulation process could only be observed visually. For the first three semi-continuous reactor studies (studies 1, 2 and 3) visual observation was the main parameter in comparing the granules in terms of size, stability and time of granulation.

3.4.2. Other Analyses

Influent and 24-hr composite effluent samples of USB reactors were analyzed for SCOD, BOD_5, SS, VSS and alkalinity, VFA contents and pH. pH measurements were performed with a pH meter (Model 2906, Jenway Ltd., UK) and a pH probe (G-05992-55, Cole Parmer Instrument Co., USA). VFA (as HAc) and bicarbonate alkalinity were measured according to the titration procedure given by Anderson and Yang (1992). BOD_5 and SS / VSS contents were measured by following standard methods (5210 B and 2540 D, E) (APHA, 1998). COD content was measured according to an EPA approved reactor digestion method (for a COD

range of 0-1500 mg/L) as given in Hach Water Analysis Handbook (1988). For COD analysis, Hach Spectrophotometer (Model 45600-02, Cole Parmer Instrument Co., USA), vials and hand-made COD solutions were used. The calibration curve of the hand-made solution with respect to the Hach Water Analysis Handbook (1998) had R^2 of 0.9933 indicating the applicability of COD solution for the analyses (Appendix B). For SCOD analyses the samples were initially filtered through 0.45 μm sterilized filter paper (Millipore).

DO concentration in the USB reactors was measured by inserting the DO probe through the second sampling port which was located at the middle height and specially designed for DO measurements (Figure 3.3). The sample withdrawn from this port was entrapped between the DO probe and the reactor, thus not contacted with the atmosphere. DO analyses of the semi-continuous reactors (study 4, Section 3.3.1.4) were performed by gently opening the rubber septa of the reactors without disturbing the liquid and immediately inserting the DO probe inside the reactor.

During SMA analyses, methane content of the daily gas production could not be measured by a gas chromatography due to the technical problems encountered. However, in order to have at least rough information about the existence of methanogens and their activities, methane content determination was accomplished by using KOH stock solution (Sawyer and McCarty, 1987). The headspace of each reactor conducted for SMA analysis was sampled with a glass gas-tight syringe; the syringe needle was inserted through the reactor septum, the headspace gas was drawn in the syringe and entrapped by a three-way valve connected to the syringe; the syringe needle was withdrawn from the reactor and inserted through the septum of an entirely closed serum bottle containing concentrated KOH stock solution (20 g/L); the three-way valve was opened and the sample was injected into the bottle at atmospheric pressure. The serum bottle was shaken manually for 4-5 minutes. CO_2 and CH_4 are the main gases that are produced during anaerobic degradation of wastes (i.e. HAc for the SMA analysis).

Thus, due to the reaction occurring between KOH and CO_2, the remaining gas in the bottle resembled CH_4 gas. CH_4 was syringed out and measured to determine the daily methane gas production.

Statistical Analysis: One-way Analysis of Variance (ANOVA) (at a significance level of 0.05) was performed to determine whether the granules/cultures developed in the reactors or their performances are statistically different. ANOVA was applied to the data of particle size analyses of semi-continuous reactors (study 4, Section 3.3.1.4) and to the calculated ratio values of SCOD removal efficiency per g VSS (of each USB reactors in study 3, Section 3.3.2.3) by using a sub-program of Microsoft Office Software Excel 98.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Semi-Continuous Reactor Experiments

In semi-continuous reactor experiments, the objective was to develop combined anaerobic and aerobic cultures in granular form from a mixture of suspended aerobic and anaerobic cultures under alternating aerobic and/or microaerobic / anaerobic conditions. Three semi-continuous reactor studies (studies 1, 2 and 3) were conducted to investigate the possibility of granulation under these conditions and factors affecting the granulation.

4.1.1. Study 1: Effect of Oxygen Dose

The aim of the first semi-continuous reactor study was to evaluate the possibility of granulation and to determine the effect of oxygen dose on granulation of mixed suspended anaerobic and aerobic seed cultures. To this purpose, five oxygen doses, namely, 10, 30, 60, 100 and 120% of the total COD added were applied to the reactors fed with either ethanol or glucose+HAc via continual two-day schedule (Figure 3.1). The reactors were maintained on continual two-day schedule for 38 days (almost four SRTs). During this period, the content of the reactors (color of the supernatant as an indicator of the redox conditions and the cultures) and sizes of the seed cultures were visually monitored. Due to the continual two-day schedule, the reactor caps could not be opened for the particle size analyses in order not to disturb the headspace gas content and in turn existing aerobic / anaerobic conditions in the reactors. Therefore, visual observation was

the main parameter in determining oxygen dose effect by comparing the developed cultures / granules in terms of size and stability and time of granulation. It should be noted that due to the same reasons (Section 3.4.1) described above, visual observation was also used in the second and third semi-continuous reactor studies.

During 38 days, the reducing and oxidizing conditions in the reactors were monitored by the color exhibited by the resazurin dye in the BM. Resazurin is clear (exhibits no color) at an oxidation-reduction potential relative to a standard hydrogen electrode (Eh) below approximately –50 millivolts (mV), but is pink under more oxidized conditions (Zitomer, 1998). DO concentrations in the anaerobic control reactors were typically zero as indicated by the colorless supernatant through the experiment. During odd days of the schedule, all the test reactors were colorless indicating the low redox conditions. Test reactors fed with oxygen doses above and equal to 60, 100 and 120% of the total COD added were typically pink most of the times during even days of the schedule. Cyclic colorless and pink supernatant colors observed in the 60, 100 and 120% oxygen-fed test reactors through odd and even days of the continual two-day schedule, respectively, indicated the achievement of reduced-oxidized conditions. Aerobic conditions were also confirmed in 10 and 30% oxygen-fed test reactors during short periods of approximately 15 minutes immediately after oxygen addition by the pink color of resazurin. Visual observations implied that all the studied oxygen doses resulted in varied periods of alternating cyclic anaerobic / aerobic (in turn their transient microaerobic) conditions in the test reactors via continual two-day schedule.

Experimental results indicated that it was possible to develop combined cultures in granular form from a mixture of suspended anaerobic and aerobic cultures via continual two-day schedule. As SS content of the reactors decreased, small sized-granules growing up to 2-2.5 mm were obtained. It was observed that mixed suspended anaerobic and aerobic seed cultures followed a granulation process

which might be described in five main phases (from Phase 0 to 4). Phases observed during granulation are given in Figure 4.1.

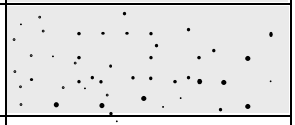
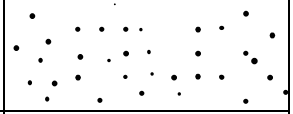
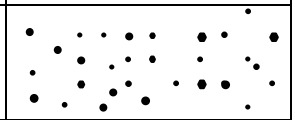
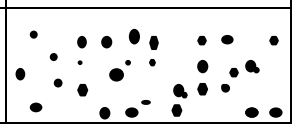
Phases observed during granulation		Schematic view of the seed cultures (not to the scale)
0	All cultures in its original suspended solid form, no pellets	
1	Mainly suspended cultures, very few pellets	
2	Notable removal of SS, pelletized cultures (loose and soft appearance) with diameter (d_p) < 0.3 mm	
3	No visible SS, supernatant is clear. $d_p \leq 1$ mm, compact structure	
4	No visible SS, supernatant is clear. $d_p \leq 2 - 2.5$ mm, compact structure	

Figure 4.1. Phases observed during granulation and schematic view of the seed cultures in each phase (Phases were determined with respect to the visual observation).

As seen in Figure 4.1, suspended cultures grew to loose floc-like pelletized cultures (Phase 2), which were then developed to compact granular cultures (Phases 3 and 4). Granules were defined as the dense microbial consortia which have compact and rigid structure (Liu and Tay, 2004). Depending on the operational conditions, the average diameter of granules varies in the range of 0.14 to 5.0 mm (Schmidt and Ahring, 1996; Peng et al, 1999; Yu et al., 2001; Liu and Tay, 2004; Hu et al., 2005a). Therefore, compact aggregates reaching to Phases 3 or 4 were referred as granules in this study. In other words, granular

cultures were defined as the ones having compact and rigid structure (unlike the floc-like pellets of loose and soft appearance) with particle size diameters above and equal to 0.1-0.2 mm. It should be noted that the diameters given in Figure 4.1 indicate the maximum sized-cultures/granules observed in the corresponding phase, which means granules of smaller sizes were also developed. The granulation processes of the seed cultures observed in each reactor fed with different oxygen dose and substrate are given in Figure 4.2.

As seen in Figure 4.2a and 4.2b, independent of the substrate type, granules up to 1 mm and 2-2.5 mm were developed (Phases 3 and 4) in all of the test reactors receiving oxygen doses of 60, 100 and 120% of the total COD added. The cultures fed with 30% of oxygen also displayed a granulation process, but limited to the formation of loose floc-like pelletized cultures. Oxygen dose of 10% was inadequate for granulation. These results implied that granulation of mixed suspended anaerobic and aerobic cultures in the same reactor was highly related with the oxygen dose applied on the second day (even days) of the schedule. Oxygen doses equal to and above 60% of the total COD added might have resulted in the formation of alternating anaerobic / aerobic conditions with adequate periods for the growth of anaerobic and aerobic cultures.

Granulation was even observed in the aerobic control reactors (independent of the substrate type) in a very short period (almost in 4-6 days), while the cultures in anaerobic control reactors were in suspended form most of the time (Figure 4.2a, b). Due to the presence and absence of granules in the aerobic and anaerobic control reactors, respectively, it might be considered that granulation in the test reactors was due to only aerobic seed cultures and their cultivation under adequate oxygen doses. However, granules in aerobic controls got smaller and had loose floc-like structure in two weeks time (Figure 4.2a, b), while, the ones in test reactors fed with same oxygen dose (120%) and substrate type as the aerobic controls did not disintegrate but remained intact till the end of the experiment.

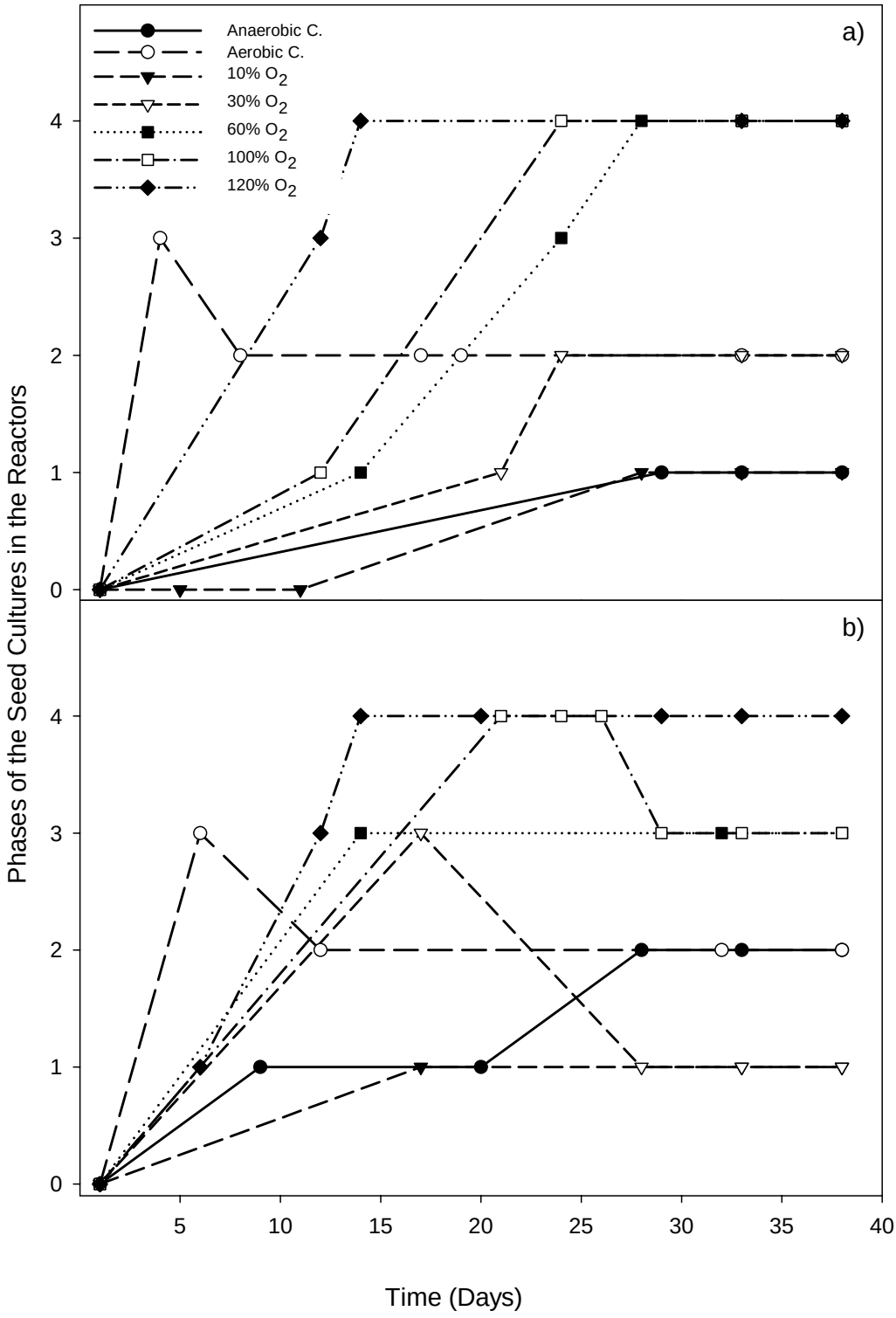


Figure 4.2. Granulation phases of the cultures in the reactors fed with a) ethanol, b) Glucose+HAc. (Definition of each phase was given in Figure 4.1)

As mentioned previously, the only difference between the aerobic controls and 120% oxygen-fed test reactors fed with same substrate type was the existence of anaerobic cultures in the test reactors in which disintegration was not observed. Therefore, anaerobic cultures might have had a significant place in the granulation process and been responsible of the intact structure of the granules developed in the test reactors. It can be stated that anaerobic cultures might have strengthened the granules developed and increased their stability. Considering the granulation process observed in aerobic controls and test reactors during the experimental period of 38 days unlike the anaerobic controls, it can also be stated that aerobic cultures might have been triggering the granulation process via the sufficient oxygen dose.

Based on the results, it can be postulated that granulation in the test reactors might be mainly related with the interaction between anaerobic and aerobic cultures, the oxygen doses applied and the schedule itself. The granulation and survival mechanisms of these two types of cultures might be explained here by a hypothesis: the formation of alternating cyclic anaerobic / aerobic and/or microaerobic conditions through the odd to even days of the schedule. Oxygen addition of the required doses (≥60%) on even days might cultivate the aerobic cultures. By the end of even days as DO content decreases and the conditions change from aerobic to microaerobic and even anaerobic conditions on odd days, filamentous bacteria might be developed, due to low DO level which triggers the flocculation (Bossier and Verstraete, 1996). After the formation of loose floc-like pellets, anaerobic cultures might locate inside these pellets for protection against aerobic conditions achieved on even days as a survival mechanism. Therefore, in one way, aerobic conditions (achieved by oxygen doses of ≥ 60%) might support the living of anaerobic cultures. A similar survival mechanism was demonstrated in gel beads, where anaerobic cultures grew mainly in the oxygen deficient central part while aerobic cultures located at the outer parts of the beads surrounded with a DO of 7 mg/L (Kurosawa and Tanaka, 1990). As Watnick and Kolter (2000)

noted, in biofilms, bacteria distribute themselves according to who can survive best in the particular environment and the high complexity of the resulting microbial community appears to be beneficial to its stability. Therefore, loose pellets where the anaerobic cultures are used as core and thus protected might turn into compact and stable granules as the cultures acclimate to the alternating conditions and operational mode. Under optimum operational conditions (i.e. substrate type, operational mode, oxygen dose etc.), the granules might develop and grow in size.

As the oxygen dose increased from 0 (anaerobic controls) to 120%, the color of the cultures or developed granules changed from dark black to light brown. The brown color of the granules (Hu et al., 2005a) might be indicative of the intensity of the aerobic cultures that were located at the outer parts of the granules and increasing with the oxygen dose applied. Independent of the substrate type used, all the test reactors fed with 60% oxygen had black-brown granules, while the ones fed with 10 and 30% oxygen had black colored cultures as the anaerobic cultures. Similarly, 120% oxygen-fed test reactors were alike the aerobic control reactors in terms of color of the granules (light brown). In all of the 100% oxygen-fed test reactors, granules of all sizes were brown in color.

In ethanol-fed test reactors, oxygen doses equal to and above 60% led to formation of large and stable granules up to 2-2.5 mm (Figure 4.2a). Time required to achieve granules of up to 2-2.5 mm diameter (Phase 4) decreased with the increase in the oxygen dose. Maximum sized-granules (2-2.5 mm) were achieved in 28, 24 and 14 days in the ethanol-fed test reactors with oxygen doses of 60, 100 and 120%, respectively. However, in glucose+HAc-fed test reactors, the largest sized-granules (Phase 4) with diameter of 2-2.5 mm were only developed in the reactors receiving 100 and 120% oxygen (in 21 and 14 days, respectively) (Figure 4.2b). The test reactors receiving 60% oxygen and glucose+HAc had granules of smaller sizes (up to 1 mm) (Figure 4.2b). Besides, granule disintegration was not observed in the ethanol-fed test reactors. However,

in the glucose+HAc-fed test reactors receiving 60% oxygen suspended solids were observed and in the ones receiving 100% of oxygen granules became smaller in size (up to 1 mm) at the end of 38 days (Figure 4.2b). Therefore, it can be stated that ethanol-fed granules were more stable compared to the glucose+HAc-fed ones. Higher stability of the ethanol-fed granules might be attributed to the higher oxygen tolerance of the anaerobic cultures located at the inner parts. Kato et al. (1993) demonstrated that ethanol provided higher oxygen tolerance than acetate for anaerobic granules.

Sludge amount wasted on the odd days of the schedule in the 10 and 30% oxygen-fed reactors which had cultures smaller than the inner diameter of needle (i.e. 1-1.25 mm) was most probably more than in the reactors having granular cultures. Wasting process performed through the odd days of the schedule might supply a competitive advantage for the granule-forming cultures, however; on the other hand, it might result in continuous sludge loss in all reactors.

4.1.2. Study 2: Effect of Substrate Type, Shaking and Sludge Wasting

The second semi-continuous reactor study was performed to determine the effects of substrate type, shaking and sludge wasting on granulation of a mixture of suspended anaerobic and aerobic cultures. Three sets of reactors, namely, Sets A, B and C, were conducted (Table 3.3, Section 3.3.1.2). In order to determine the effect of shaking on granulation, the reactors in Set A (shaking, but no wasting) were compared with the ones in Set B (no shaking and no wasting). Besides, the reactors in Set B were compared with the ones in Set C (no shaking, but wasting) to determine the effect of sludge wasting on granulation. The two oxygen doses (60 and 120%) applied to each set were selected according to the results of the first semi-continuous reactor study (Section 4.1.1) indicating the oxygen doses of 60, 100 and 120% as the doses leading to granulation. Four substrates, namely,

ethanol, glucose, HAc and glucose+HAc were applied to each set. The experiments lasted 28 days.

Because of the high amount of seed content in the test reactors (16990 ± 247 and 7842 ± 106 mg/L of MLSS and MLVSS, respectively) and no wasting process in Sets A and B; it was hard to observe the granulation process during the experimental run. Therefore, it was planned to investigate the effect of initial seed content on granulation in the next semi-continuous reactor study.

Unlike the test reactors, aerobic control reactors could easily be observed for granulation due to their low aerobic seed content (i.e. 1440 ± 21 mg/L MLSS and 1025 ± 19 mg/L MLVSS). The first granules in the aerobic control reactors of Sets A and B were observed on Days 2-4 and 10, respectively. In addition, the granules obtained in Set A (up to 2.5 mm in diameter) were larger and more in number than the ones in Set B (up to 1 mm). Thus, shaking process was postulated to decrease the period required for granulation via increasing the gas-liquid interfacial area, the oxygen transfer rate (Field et al., 1995) and in turn the growth of cultures.

Granulation was not observed in the reactors of Set C, where sludge wasting process was applied unlike Set B. The loss of the seed cultures in all reactors (Set C) was clearly detected throughout the experiment and almost no seed culture remained in the aerobic control reactors of Set C at the end of the experiment. No granulation was observed in the test reactors. It is obvious that sludge wasting process applied through the continual two-day schedule (Figure 3.1) resulted in the continuous loss of the seed culture. However, the first study (Section 4.1.1) indicated that granules could be developed in the test reactors (60 or 120% oxygen) despite of wasting process when shaking was applied. Therefore, it can be said that application of wasting process without shaking might lead to the loss of the cultures that might start the granulation. However, on the other hand, sludge wasting might supply a competitive advantage for the granule-forming cultures by

removing the ones not occupying in the granulation and in turn increasing the substrate and oxygen load that the granule-forming cultures are exposed to.

In order to compare the effect of substrate type on granulation, aerobic control reactors of Set A and Set B, in which granulation was observed, were considered. Granulation was observed in all of the aerobic control reactors independent of the substrate type indicating the granulation was mainly accomplished with the continual two-day schedule and the oxygen dose applied (here 120%). However, substrate type affected the granulation period, the amount of the granules and their sizes. Granules were developed in the reactors fed with, in the order of, glucose, glucose+HAc, HAc and ethanol. However, as the granules in the reactors fed with ethanol grew to larger sizes, the ones in the reactors fed with other substrates got smaller, disintegrated and SS amount in these reactors increased through the end of the experiment. The similar disintegration was also observed for glucose+HAc and 100% oxygen-fed granules in the test reactors of the first semi-continuous reactor study (Figure 4.2b). Therefore, among the four substrates, ethanol resulted in the formation of the largest and most stable granules for both of the sets. The result of more stable granulation under ethanol feeding was consistent with the results of the first study.

4.1.3. Study 3: Effect of Initial Anaerobic to Aerobic Biomass Ratio and Total Biomass Concentration

The results of the first two studies (studies 1 and 2) revealed that anaerobic to aerobic MLVSS ratio (R-value) and initial total biomass concentrations might have affected the granulation process. Therefore, in order to determine the effects of R-values and the optimum R-value leading to granulation, five R-values, namely, 1.7, 2.9, 4.6, 5.7 and 7.5 were studied in this study (Table 3.4, Section 3.3.1.3). The test reactors fed with ethanol and 120% of oxygen dose were

maintained on continual two-day schedule with shaking (Figure 3.1, but without wasting and consequent BM adding process) for 42 days.

It was observed that sludge bed in the test reactors having MLSS content of almost 7800 mg/L had a layered structure in terms of color changing from brown to black as going from top to the bottom. The sludge bed color in these test reactors turned into brown completely till the end of the experiments. This might be attributed to the shaking process, diffusion of oxygen and in turn the growth of aerobic cultures. On the other hand, the color of the sludge bed in the test reactors with MLSS content of almost 16000 mg/L was almost black most of the time. Due to the high MLSS content and in turn limited oxygen transfer, the formation of layered sludge bed (brown-black) slowly proceeded towards the end of the experiment.

Granulation was observed in all of the test reactors with low initial MLSS content. However, granulation process could not be visually observed in the ones with high initial MLSS content due to the dense sludge bed. Therefore, at the end of the experimental run, the caps of the test reactors were opened and their contents were examined for granule formation. It was observed that R-values did not affect the maximum granule sizes. Independent of the R-value, granules of almost 2-2.5 mm were developed in the test reactors with initial MLSS concentrations of 7800 mg/L. However, the test reactors with same R-value but higher MLSS content (almost 16000 mg/L) had pelletized-cultures which were smaller in size ($\leq$ 0.5 mm) and fewer in number. Development of granules with smaller sizes might be due to the high seed content, which might have been a barrier to oxygen transfer and provided resistance to its mass transport (Field et al., 1995). Such high initial seed sludge content (equal to or above 16000 mg/L) and not applying wasting process (as in this study) through the continual two-day schedule might have prevented the formation of alternating cyclic anaerobic / aerobic and/or microaerobic conditions. This might have prevented the formation of the optimum conditions for each culture type and in turn their growth.

4.1.4. Study 4: Determination of Physical and Microbial Characteristics

Based on the results of the first three semi-continuous reactor studies, a set of semi-continuous reactors was designed and operated. The aim was to develop combined cultures from a mixture of suspended anaerobic and aerobic cultures in granular form under alternating anaerobic / aerobic and/or microaerobic conditions. It was also planned to investigate the developed granules in terms of their physical (settling velocity, particle size) and microbial (SOUR and SMA) properties.

Continual two-day schedule applied in this study (Figure 3.2, Section 3.3.1.4) was modified considering the results of the first three semi-continuous reactor studies. As seen in Figure 3.2, ethanol was the carbon source in this final set, because it resulted in the formation of the largest and most stable granules in the first and second studies (Sections 4.1.1 and 4.1.2). The first study indicated that the oxygen doses leading to granulation were 60, 100 and 120% of the total COD added (23, 38 and 45 mL, respectively), when the substrate was ethanol (Section 4.1.1). Therefore, in this part of the study, the minimum (60%, 23 mL) and maximum (120%, 45 mL) oxygen doses leading to granulation were applied in order to examine the effect of extremist doses on the microbial structure of the granules and in turn their microbial activities (SOUR and SMA). Moreover, although the previous studies indicated that wasting of the sludge resulted in continuous sludge loss, sludge wasting was also practiced in this study due to the fact that it supplies a competitive advantage for the granule-forming cultures. In addition, the reactor content, which might be a barrier to oxygen supply and prevent its transport, might be diluted by sludge wasting and BM adding process. This might speed up the granulation because granule-forming cultures would be more likely to contact with DO and substrate. The wasting process was accomplished with a syringe and needle with inner diameter of 0.5 mm to achieve selective pressure in the reactor (i.e. the removal of the loose pellets with diameter less than 0.5 mm) and to prevent the loss of the cultures that might start granulation.

All reactors, namely, anaerobic and aerobic control, 60% and 120% oxygen-fed test reactors were maintained on continual two-day schedule for 68 days (almost 7 SRTs). DO concentrations were measured to investigate whether alternating aerobic and/or microaerobic / anaerobic conditions were achieved in the reactors via the continual two-day schedule (Table 4.1). As seen in Table 4.1, 60% oxygen-fed test reactors displayed alternating cyclic conditions from anaerobic (DO: 0 mg/L, odd days) to microaerobic/aerobic (DO: 0.8-3.1 mg/L, even days). Due to greater amount of oxygen addition, 120% oxygen-fed test reactors displayed microaerobic conditions (rather than anaerobic) on odd days (DO: 0.5-1.1 mg/L) and aerobic conditions on even days (DO: 5.2-6.3 mg/L). But it should be noted that due to the limitation of oxygen diffusion (De Beer et al., 1993; Lens et al., 1995; Okabe et al., 1999; Peng et al., 1999; Meyer et al., 2003) anaerobic conditions could also be achieved in 120% oxygen-fed reactors at the inner parts of the granules once they were developed (Section 4.1.4.3). Therefore, proposed alternating anaerobic / aerobic and/or microaerobic conditions could be reached. Table 4.1 also indicates that the required anaerobic and aerobic conditions were achieved in the anaerobic and aerobic control reactors, respectively.

Table 4.1. DO concentrations in the reactors measured during continual two-day schedule.

Reactor type	DO (mg/L)	
	Day 1 (odd days)	Day 2 (even days)
Anaerobic control	0.0	-
Aerobic control	-	6.0 - 7.8
60% oxygen-fed test	0.0	0.8 - 3.1
120% oxygen-fed test	0.5 - 1.1	5.2 - 6.3

4.1.4.1. Granulation Process in Semi-Continuous Reactors

Experiments revealed that granulation of a mixture of suspended anaerobic and aerobic cultures was possible with the continual two-day schedule. Granules with median diameter of up to 1.28 and 1.86 mm were developed in 60 and 120% oxygen-fed test reactors at the end of 68 days, respectively. Granulation was also observed in aerobic control reactors, which was consistent with the results of the previous semi-continuous reactor studies. Although slowly growing granules of 0.5 mm were observed in the anaerobic control reactors at the end of 68 days, the seed cultures were in suspended form most of the time. Therefore, time dependent development of the granules in terms of median diameter was only illustrated for 60 and 120% oxygen-fed test and aerobic control reactors (Figure 4.3a, b and c, respectively). The deviations on each datum given in Figure 4.3 indicate the sizes of granules with minimum- and maximum-sizes.

To illustrate the existence of combined cultures / granules and their structures the image analyses were performed for each reactor type and illustrated in Figure 4.4. From this part forward, the granules developed in the anaerobic and aerobic control reactors were defined as “anaerobic” and “aerobic granules”, respectively, while the ones in test reactors as “combined granules”.

Through the granulation process suspended cultures changed to floc-like sludge and pellets and then gradually developed to compact granules with rigid structure. The floc-like sludge around the granules developed in the 60 and 120% oxygen-fed test reactors can be clearly seen in Figure 4.4f and h, respectively.

The granulation process observed in the 60% oxygen-fed test reactors and their images are given in Figure 4.3a and Figure 4.4f and g, respectively. The seed cultures in suspended form grew to a size of 0.4 mm in 16 days (Figure 4.3a). Granules with median particle sizes of 0.4 mm gradually developed and reached to a size of 1.28 mm at the end of experiment with rigid and compact structure.

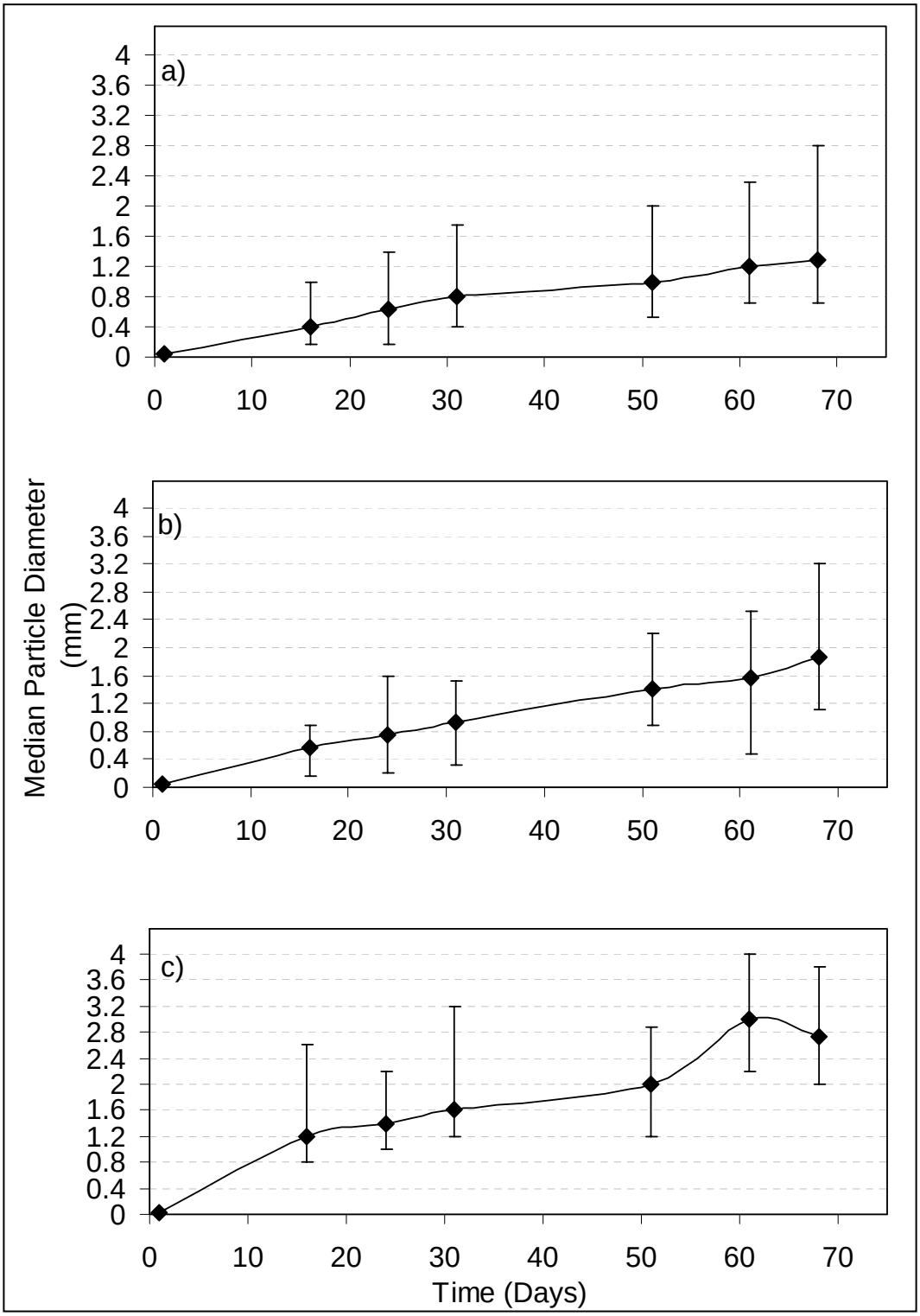


Figure 4.3. The changes in the median diameters of the granules developed in the, a) 60% oxygen-fed test, b) 120% oxygen-fed test, c) aerobic control reactors.

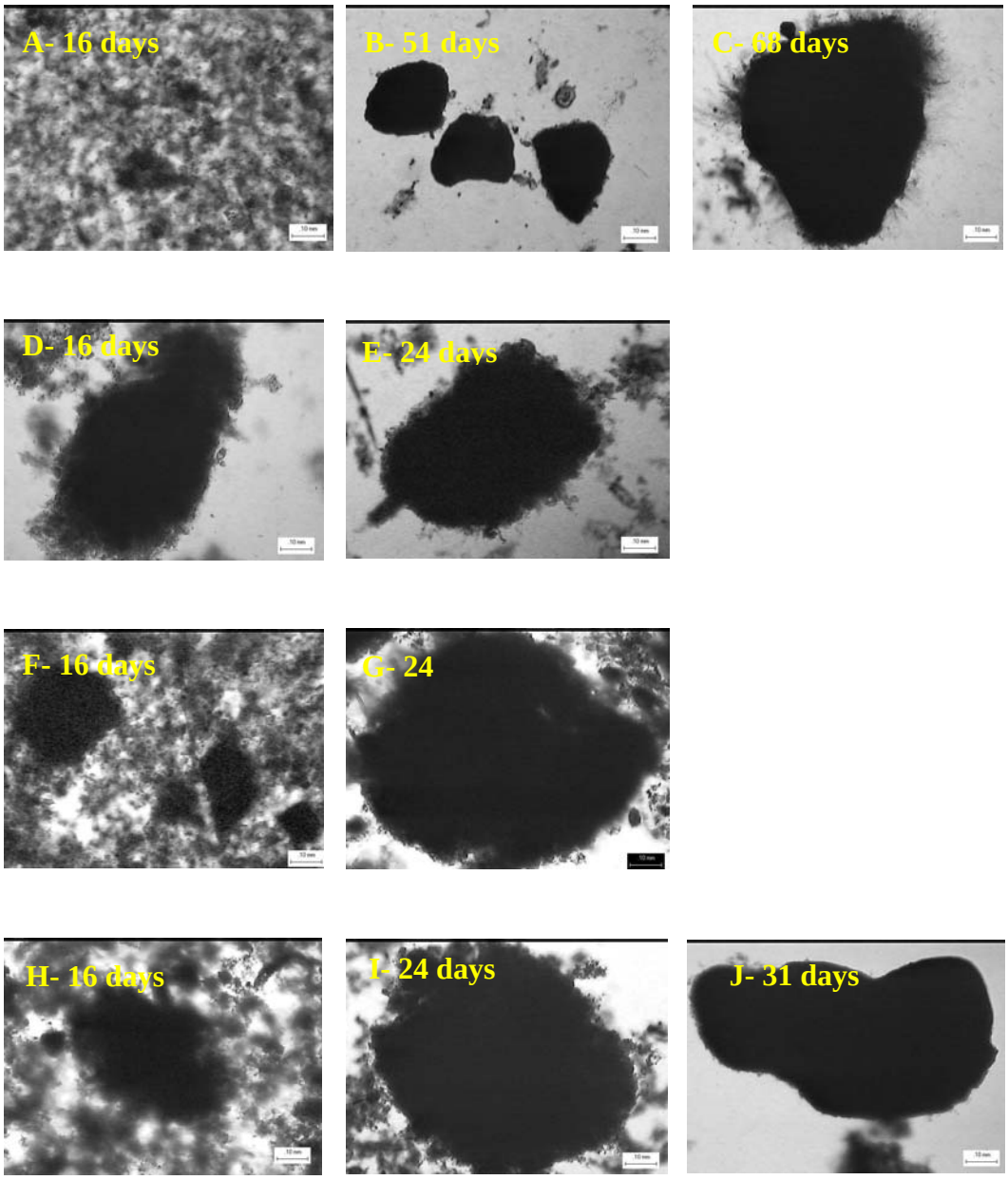


Figure 4.4. Images of the cultures / granules in anaerobic control (a, b, c), aerobic control (d, e), 60% oxygen-fed test (f, g) and 120% oxygen-fed test reactors (h, i, j). Images were taken on Day 16 (a, d, f, h); on Day 24 (e, g, i); on Day 31 (j); on Day 51 (b); on Day 68 (c). (Bar = 0.1 mm)

The similar increasing trend in granule sizes was also observed in the 120% oxygen-fed test reactors (Figure 4.3b). The granules with median particle sizes of 0.58 mm (on Day 16) grew up to 1.86 mm by the end of 68 days. Visual observations indicated that the color of the combined granules fed with 60% oxygen changed from black (as anaerobic cultures) to brown, while the 120% oxygen-fed combined granules from brown to light brown through the operation period. Combined granules fed with 120% oxygen had also compact structures as the 60% oxygen-fed ones. However, 120% oxygen-fed granules developed a fluffy surface towards the end of the experiments, which was also observed on the aerobic granules developed in the control reactors (Figure 4.4d and e). Fluffy surface might have been related with the aerobic cultures and high DO conditions and occurred due to the ECP production (Hu et al., 2005a) and/or the proliferation of filamentous bacteria on the granule surface (Shen and Quiot, 1996) at high DO.

ANOVA (Appendix D) performed for the particle size data indicated that combined granules fed with 60 and 120% oxygen developed through the operation period (in turn the granulation process) were statistically different from each other. When the granulation process was considered, it was observed that the growth rate of the combined granules fed with 120% oxygen was higher than the granules fed with 60% oxygen (Figures 4.3a and b). Besides, maximum-sized combined granules fed with 120% oxygen were 3.2 mm, while it was 2.8 mm in the 60% oxygen-fed granules. This might be explained by the granulation mechanism: the granules developed in the test reactors might be composed of anaerobic and aerobic cultures, which was verified by the SMA / SOUR analyses which will be provided in the following parts (Section 4.1.4.3). Aerobic cultures might have been located at the outer parts of the granules, while, anaerobic seed cultures might have been located at the inner parts as a survival mechanism. The presence of strictly anaerobic cultures deep inside the original aerobic activated sludge flocs and aerobic granules was demonstrated (Wu et al., 1987; Tay et al., 2002a), which supports the hypothesized survival and in turn the granulation mechanisms. The higher oxygen dose applied for the 120% oxygen-fed test

reactors might have led to cultivation of greater amount of aerobic cultures at the outer parts of the granules and in turn development of larger granules in shorter periods compared to 60% oxygen-fed ones.

Granulation was observed in the anaerobic and aerobic control reactors (Figures 4.3c and 4.4b, c). However, the granules of 0.5 mm (compact structure and black) in anaerobic control reactors were developed towards the end of the experiments (in 68 days) and were fewer in number (Figures 4.4b and c). Granulation in anaerobic control reactors was not observed before in the previous semi-continuous reactor studies most probably due to the duration of the experiments which was less than 50 days. On the other hand, granules of 0.5 mm (fluffy surface and light brown in color) were developed in almost 5 days in the aerobic control reactors (Figure 4.3c). Granulation rate of the aerobic granules was greater than the combined granules (Figure 4.3). Aerobic granules with median size of 1.2 mm were developed at the end of 16 days, which was almost three-fold and two-fold larger than the combined granules fed with 60 and 120% oxygen, respectively. At the end of 68 days, aerobic granules reached to a median size of 2.72 mm with maximum sizes of 3.8 mm. The greater growth rate of aerobic granules compared to the combined granules might be due to the substrate loading per gram of aerobic bacteria (as VSS). The aerobic control reactors differed from 120% oxygen-fed test reactors by the absence of anaerobic seed cultures. Therefore, the substrate loading in aerobic control reactors was channeled only to the aerobic cultures resulting in higher amount of aerobic cultures to cultivate and in turn larger granules with higher growth rates.

Considering the granules developed in all four types of reactors, it can be postulated that granulation was highly dependent on the peculiarity of the continual two-day schedule. The granulation of mixed suspended anaerobic and aerobic cultures in the test reactors was associated with the sufficient oxygen dose applied and the alternating cyclic anaerobic / aerobic and/or microaerobic conditions of adequate periods achieved by the continual two-day schedule.

Granulation was observed in the test reactors fed with oxygen doses above and equal to 60% oxygen of the total COD added (Section 4.1.1). Therefore, it was the oxygen dose (i.e. sufficient amount of oxygen) leading to alternating cyclic (DO) conditions of adequate periods which triggered the granulation of anaerobic and aerobic cultures in the test reactors. The shaking (mixing) and wasting processes (that speeded up the granulation) or the substrate type (that affected the size and the strength of the granules) were not the factors that triggered the granulation of mixed suspended anaerobic and aerobic cultures (Sections 4.1.1, 4.1.2 and 4.1.3).

Through even to odd days of the two-day schedule, DO content of the test reactors decreased gradually leading to the alternating conditions from aerobic to microaerobic (as in 120% oxygen-fed tests) and from aerobic to microaerobic and anaerobic conditions (as in 60% oxygen-fed tests) (Table 4.1). At low DO level, filamentous bacteria might be developed, which triggers flocculation (Bossier and Verstraete, 1996). As soon as the loose floc-like pellets are formed, anaerobic cultures might locate inside these pellets for protection against aerobic / microaerobic conditions as a survival mechanism. At high DO, more ECPs are produced (Peng et al., 2001) by which floc-formers might attach to the flocs/pellets (Cenens et al., 2002) and enlarge the size. ECPs (especially extracellular polysaccharides), which are highly hydrated gel acting as a cementing substance (De Beer et al., 1996), play an important part in maintaining the formation and structural integrity of the granules (Tay et al., 2001c). Therefore, granulation period might be mostly determined by outer-locating aerobic cultures. As cultures go through the alternating cyclic DO conditions and adapt to these conditions, and cultivate, these pellets using the anaerobic cultures as core might turn into compact and strong granules. Agglomeration is the respond of the microorganisms against environmental stresses (physical or chemical) done to be sheltered (Bossier and Verstraete, 1996). It has been demonstrated that co-aggregation mediated interactions between an obligate anaerobe *Fusobacterium nucletuatum* and other oxygen-tolerant species

(planktonic phases of a complex community of oral bacteria) facilitated the survival of anaerobes in aerated environments (Bradshaw et al., 1998).

Bacteria in a multispecies biofilm are not randomly distributed but rather organized to best meet the needs of each (Watnick and Kolter, 2000). They distribute themselves according to which can survive best in the particular microenvironment and also based on symbiotic relationships between the groups of bacteria (Bradshaw et al., 1998; Okabe et al., 1999; Watnick and Kolter, 2000). Consequently, the distribution of different microbial populations in a granule may have an effect on its stability (Liu and Tay, 2004). Therefore, as well as anaerobic cultures, location of anaerobic cultures at the oxygen-free core of the pellets developed in test reactors might also be advantageous for aerobic cultures in terms of increasing the stability. First semi-continuous reactor study (Section 4.1.1) indicated that aerobic granules developed in the aerobic control reactors disintegrated in 8-12 days, while the combined granules faced with same environmental conditions and operational processes did not disintegrate and remained intact after 38 days (Figure 4.2). Besides, the settling velocity of the aerobic granules developed in this study (Section 4.1.4.2) was lower than those of the combined granules despite of the greater sizes. The increased stability, and denser and more compact structure of the combined granules compared to the pure aerobic granules might be attributed to the anaerobic cultures located inside. Liu et al. (2004) stated that selecting slow-growing bacteria in aerobic granules improved the stability of the granules. The high growth rates encouraged the outgrowth of aerobic granules leading to a rapid increase in the size of granule, but further a loose structure with low biomass density and reduced stability. They further stated that a practical strategy for improving the stability of aerobic granules can be achieved by manipulating operational conditions or through selecting slow-growth bacteria. This was achieved in this study by combining anaerobic and aerobic cultures via continual two-day schedule.

The adequate oxygen dose (i.e. amount) and alternating cyclic (DO) condition-triggered granulation mechanism, which was postulated for the combined granulation in test reactors, also explains the granulation in aerobic control reactors. Because oxygen was applied to the aerobic control reactors only in the second day (even days) of the schedule, oxygen gradient was also expected through the continual two-day schedule. In aerobic granulation studies performed in SBRs, high shear force and cycle time (leading to enhanced extracellular polysaccharide production), and substrate starvation and intermittent feeding (leading to increased cell surface hydrophobicity) are given as the significant factors of cell-to-cell interaction strength and in turn granulation (Tay et al., 2001a, 2001b; Liu and Tay 2002; Liu and Tay 2004). Based on the granulation studies achieved under varied oxygen levels as 0.7-1.0 mg/L (Peng et al., 1999, 2001) or > 2.0 mg/L (Liu and Tay, 2004), DO level or gradient has not been considered as a decisive parameter for aerobic granulation in SBRs. However, DO values in these studies refer to the conditions during aeration period but not the values of whole operation cycle including filling, settling, draw and idle periods. It should be taken into consideration that after the aeration period, DO in the SBR content decreases resulting in the alternating conditions (DO gradient) through the cycles. Peng et al. (1999) achieved development of aerobic granules in SBRs under low DO (0.7-1.0 mg/L). However, DO content of the reactor up to the aeration period was given as nearly zero, which indicates the alternating conditions (anaerobic to almost microaerobic). In addition, development of aerobic granules was demonstrated in SBRs operated with cycles including anaerobic or anoxic periods of 1.5-2 hr (Zhu and Wilderer, 2003; Jang et al., 2003). These might verify the proposed granulation mechanism for aerobic cultures in the aerobic control reactors. Therefore, in determining the factors triggering the aerobic granulation in SBRs, alternating conditions (or DO gradient) prevailing in the SBR content (i.e. DO change through the cycles) should also be considered. More detailed analyses are required in order to define the mechanisms of aerobic granulation in the control reactors. However, this is beyond the scope of the thesis. Independent of the mechanisms involved,

combining anaerobic and aerobic cultures led to formation of denser, more compact and stable granules than the aerobic granules developed under the same operational conditions.

Development of granules in the anaerobic control reactors can also be related with the peculiarity of the schedule. It should be noted that anaerobic granules (of almost 0.25 mm) were developed by Day 51 (Figure 4.4b). However, as already mentioned this was not experienced before in the previous semi-continuous reactor studies most probably due to the duration of the experiments which was less than 50 days (Sections 4.1.1, 4.1.2 and 4.1.3). In the previous studies, shaking and wasting processes decreased the granulation period via increasing the gas-liquid interfacial area, the oxygen transfer rate and in turn the growth of cultures (Sections 4.1.2 and 4.1.3). However, these results were obtained by comparing the sizes of the aerobic granules or combined granules but not those of anaerobic ones due to their absence during the studied experimental periods. In anaerobic control reactors, shaking process might have resulted in particle-particle collision and wasting process might have led to hydraulic selective pressure in the reactor both leading to agglomeration (Tay et al., 2001b). However, in order to comment on the effects of shaking and/or wasting processes on anaerobic granulation, previous studies should have been extended to beyond 50 days. To define the parameters leading to granulation of anaerobic cultures required detailed analyses (Section 2.2.1). These were beyond the scope of the thesis and thus not investigated. It is for sure that the period required for anaerobic granulation (50-68 days) is much longer than the time required for granulation of combined cultures (<16 days).

Visual observations and MLSS / MLVSS analyses (Figure 4.5) performed for the liquor wasted on the odd days of the schedule indicated that suspended solid contents of the test reactors decreased suddenly in the first two weeks parallel to the granulation process (Figure 4.3).

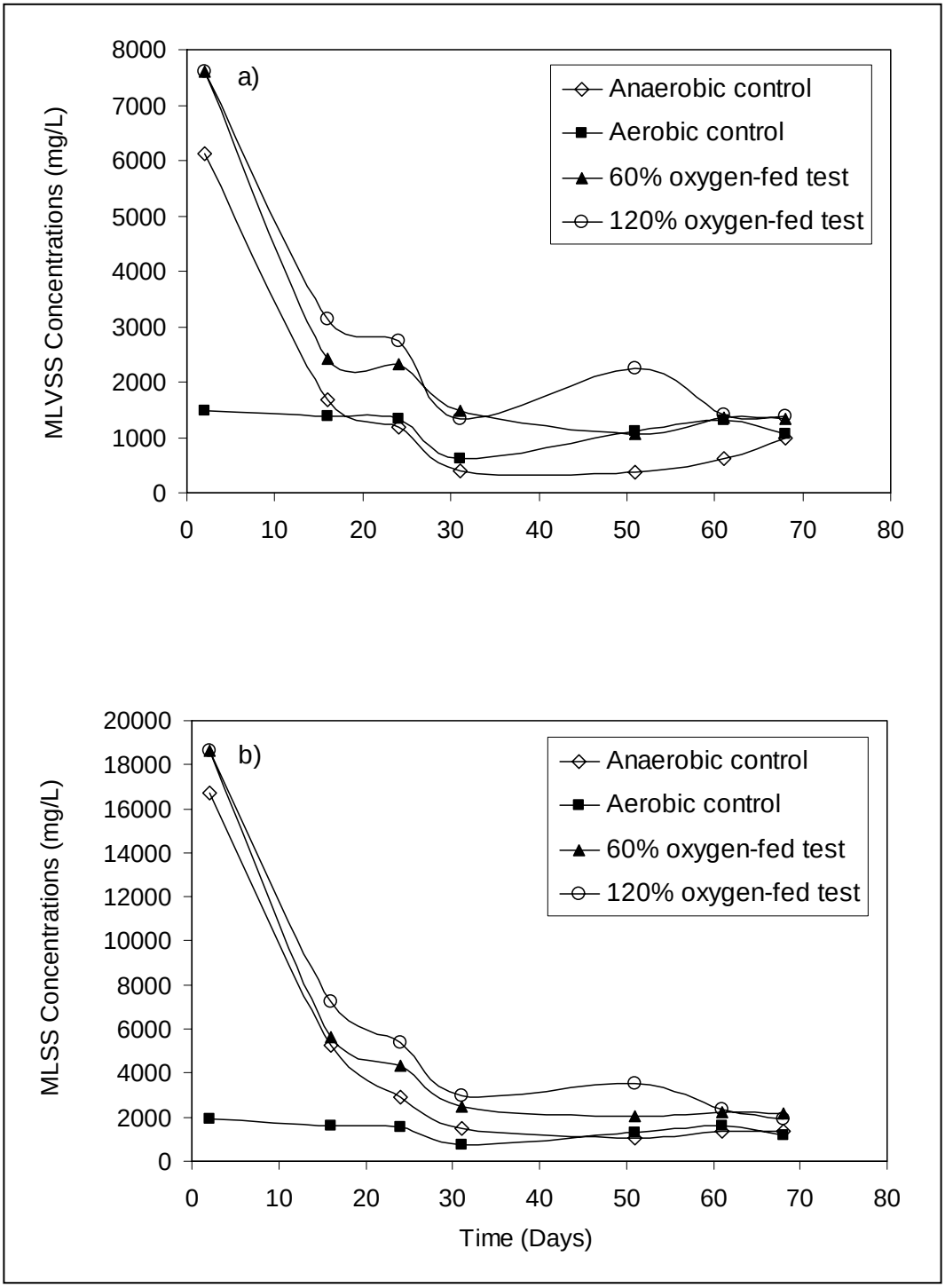


Figure 4.5. The changes in the a) MLVSS and b) MLSS concentrations of the wasted liquor of the reactors.

MLSS and MLVSS values (Figure 4.5) referred to the wasted liquor or suspended cultures that had smaller particle sizes than the inner diameter of the syringe needle (0.5 mm) and thus wasted on the first day of the schedule. On the other hand, the cultures granulated and had greater particle sizes were not washed out the reactor by the wasting process. The wasted cultures referred to the ones that did not participate in granulation. In other words, the decreasing trend of the concentration of the wasted cultures was attributed to the formation of granules in the reactors, their acclimation to the cyclic operational conditions and in turn growth in size (Wang et al., 2004; Hu et al., 2005a).

The MLSS / MLVSS contents of all reactors displayed a sharp decreasing trend within the first two week (Figure 4.5). This period was parallel to the time required for the development of combined granules with median sizes of 0.4 and 0.58 mm in the 60 and 120% oxygen-fed test reactors, respectively (Figures 4.3a and b, Day 16). MLVSS concentration slightly decreased till Day 31, after which it remained almost constant for almost 40 days. This is reasonable because the granules developed in the test reactors had already reached to median sizes greater than the inner diameter of the syringe needle (0.5 mm) by Day 31 (granules of 0.8 and 0.96 mm in 60 and 120% oxygen-fed test reactors, respectively) (Figures 4.3a and b). Figure 4.5 also indicates that the steady-state conditions in terms of effluent MLSS / MLVSS concentrations were achieved in the test reactors after Day 31. In spite of the steady-state conditions with respect to the MLSS / MLVSS concentrations, the increase in the median sizes of the combined granules was observed as seen in Figures 4.3a and b. The attachment of the suspended cultures onto the developed granules may result in the growth of the particle sizes of the granules.

The greater and continuing MLVSS loss in the anaerobic control reactors compared to the test reactors was mainly due to the slowly proceeding granulation (towards Day 51). As seen in Figure 4.5a, MLVSS content of aerobic control

reactors remained more or less constant throughout the operation period, which might be explained by the development of granules of 0.5 mm in almost 5 days (i.e. rapid granulation process) (Figure 4.3c).

4.1.4.2. Physical Characteristics of Combined Granules Developed in Semi-Continuous Reactors

At the end of 68 days, the combined granules fed with 60 and 120% oxygen reached to median sizes of 1.28 mm (with a range of 0.72 - 2.8 mm) and 1.86 mm (with a range of 1.12 - 3.2 mm), respectively. These values were comparable to the particle size ranges of the anaerobic granules and aerobic granules reported in literature as 0.l4-5 mm (Schmidt and Ahring, 1996) and 0.2-5 mm (Liu and Tay, 2004), respectively.

The average settling velocities of the combined granules developed at the end of the experiments were 31.0 ± 7.4 and 39.2 ± 8.3 m/hr for 60 and 120% oxygen-fed ones, respectively. These values were comparable to those of aerobic granules reported in literature as 30-40 m/hr (Morgenroth et al., 1997) and 22-77 m/hr (Liu and Tay, 2004; Hu et al., 2005a) and were slightly greater than the ones developed in the aerobic control reactors (i.e. 27.7 ± 6 m/hr). Besides, the settling velocities of the combined granules were comparable to those of satisfactorily settling anaerobic granules which are 20-50 m/hr (Schmidt and Ahring, 1996). Physical properties indicated that combined cultures developed from a mixture of anaerobic and aerobic cultures in the test reactors had properties as the granular cultures for both of the oxygen doses (60 or 120%) investigated.

Although the aerobic granules developed at the end of 68 days had greater median sizes of 2.72 mm (with a range of 2 - 3.8 mm), they had lower settling velocities compared to the combined granules. This was related to the low density of the granules which allowed high void space and the increase of the granules` particle

sizes (Batstone and Keller, 2001). Due to the limitations in oxygen diffusion (or nutrient) to the depths of the granules, interior biomass might die and be consumed by outer bacteria leading to holes inside the aerobic granules (Hu et al., 2005a). Lower settling velocity and in turn density of aerobic granules can also be attributed to the growth rate of aerobic cultures. It was found that high growth rate encouraged the outgrowth of aerobic granules leading to rapid increase in the size but further a loose structure and low density (Liu et al., 2004). High growth rate was found to reduce the strength of the granules i.e. partial loss of structural integrity and disintegration would occur (Tijhuis et al., 1995; Kwok et al., 1998).

Despite of the smaller sizes than those of aerobic granules, the greater settling velocity values of the combined granules fed with same oxygen dose (120%) might be attributed to the existence of anaerobic cultures in the inner parts of the combined granules leading to more compact and denser structure. This was also confirmed by SMA analyses (Section 4.1.4.3). Due to their slow-growth rate and physiological properties, anaerobic cultures located at the oxygen-deficient parts of the combined granules would not be affected by the nutrient or oxygen limitation. Therefore, voids or holes were not expected inside the combined granules due to the existence of slow-growth anaerobic bacteria, which might increase the stability of combined granules (compared to aerobic granules). Liu et al. (2004) stated that selecting slow-growing nitrifying bacteria in aerobic granules improved the stability of the granules and resulted in granules of strong structure with good settleability. The distribution of different microbial populations in a granule appears to be beneficial to its stability (Liu and Tay, 2004).

As a result it could be stated that both anaerobic and aerobic seed cultures (i.e. combined granules) should be used to develop more compact and stable granules (than aerobic granules) in shorter periods (than anaerobic granules) under the experimental conditions studied.

4.1.4.3. Microbial Activity of Combined Granules Developed in Semi-Continuous Reactors

The SOUR and SMA values of the granules developed in the reactors during 68 days were given in Tables 4.2 and 4.3, respectively. SOUR analyses were carried out in duplicates by using the reactor contents in portions. The values of coefficient of variation (i.e. standard deviation×100/average value) calculated for SOURs were in the range of 0.08-8.54%, indicating the statistically dependable data.

Table 4.2. The results of the SOUR analyses conducted for three types of reactors.

Reactor type	SOUR values (mg DO/g VSS.hr)					
	Day 16	Day 24	Day 31	Day 51	Day 61	Day 68
Aerobic control	20.55	11.17	19.38	7.24	16.40	24.99
60% oxygen-fed test	23.35	23.30	27.19	15.60	11.27	33.25
120% oxygen-fed test	25.58	6.43	9.54	7.16	10.71	47.01

As seen in Table 4.2, combined granules fed with 60 and 120% oxygen had SOUR values ranging from 11 to 33 and from 6 to 47 mg DO/g VSS.hr, respectively. These values were lower than those of the aerobic granules reported by Tay et al. (2001a) which are in the range of 55.9-69.4 mg DO/g VSS.hr. However, they were well comparable to the values of aerobic granules developed in the aerobic control reactors ranging from 7 to 25 mg DO/g VSS.hr.

Table 4.3. The results of the SMA analyses conducted for three types of reactors.

Reactor type	SMA values (mL CH_4/g VSS.hr)					
	Day 16	Day 24	Day 31	Day 51	Day 61	Day 68
Anaerobic control	18.55	25.77	37.32	80.00	99.07	67.76
60% oxygen-fed test	16.67	16.89	13.98	42.12	40.55	25.36
120% oxygen-fed test	16.22	19.19	30.99	29.59	38.38	33.48

SMA values of the combined granules fed with 60 and 120% oxygen were in the range of 14-42 and 16-38 mL CH_4/g VSS.hr, respectively, which were comparable to the values reported in literature as 16.1-57.5 mL CH_4/g VSS.hr for anaerobic granules grown on ethanol (Dolfing and Mulder, 1985; Grotenhuis et al., 1991). SMA results clearly indicated that combined granules produced significant amount of methane, demonstrating the existence and survival of the methanogens in the granules.

The noteworthy SOUR and SMA of the granules developed in test reactors verified that they were composed of both anaerobic and aerobic cultures. In spite of the alternating anaerobic / aerobic and/or microaerobic conditions (Table 4.1), the survival and noteworthy activities of the anaerobic and aerobic cultures constituting the combined granules might support the hypothesized granulation mechanism: location of the cultures based on their physiological characteristics, i.e. attachment of the aerobic cultures over the anaerobic ones. Anaerobic cultures survived by locating deep inside the granules where the oxygen could not diffuse (Beunink and Rehm, 1988, 1990; Kurosawa and Tanaka, 1990; Meyerhoff et al., 1997; Tay et al., 2002a). The penetration depth of oxygen is not only a function of the uptake rate and bulk substrate concentration, but also a function of the

duration of the aerobic period (Meyer et al., 2003). However, oxygen rarely penetrates more than a few hundred micrometers due to relatively rapid oxygen uptake compared to slow diffusion. Meyer et al. (2003) indicated that oxygen did not penetrate further than 100 μm through the granules exposed to air-saturated conditions (DO = 8 mg/L) for two hours. The oxygen diffusive layer even for the loose activated sludge flocs was given as 200-400 μm (Lens et al., 1995). Therefore, once the granules were developed, the anaerobic cultures located inside the granules were protected for both of the oxygen dose applied (60 or 120%). The fast granulation observed in the 120% oxygen-fed test reactors (Figure 4.3b) indicated the well-protection of the anaerobic cultures and thus noteworthy SMA values despite of the microaerobic to aerobic conditions prevailing in the reactor content (Tables 4.1 and 4.3).

Oxygen dose (23 mL) fed to the combined granules in the 60% oxygen-fed test reactors was half of the oxygen volume fed to the 120% oxygen-fed test reactors (45 mL). Despite of that, 60% oxygen-fed combined granules displayed SOUR activities (11-33 mg DO/g VSS.hr) that were not so distinct but even in the same range as those of 120% oxygen-fed combined granules (6-47 mg DO/g VSS.hr). It is stated that a higher affinity for oxygen utilization (i.e. decreased half saturation coefficients for the consumption of oxygen) could be observed with the decrease of initial DO concentrations from 1 to 0.05 mg/L (Yerushalmi et al., 2002). The 60% oxygen-fed combined granules or the aerobic cultures in these granules were exposed to prolonged microaerobic and even anaerobic conditions compared to 120% oxygen-fed ones. Thus, they responded metabolically to the environmental changes (stresses such as low DO) by increasing their oxygen uptake rate (OUR) activities (Figure 4.6). A similar environmental stress such as higher selection pressure (e.g. a shorter settling time) resulted in a remarkable increase in the oxygen uptake values of aerobic granules developed in SBRs (Qin et al., 2004). The greater OUR values of the 60% oxygen-fed combined granules and smaller particle sizes (i.e. low VSS in each granule) of the 60% oxygen-fed combined

granules, therefore explain their comparable SOUR activities with those of 120% oxygen-fed ones.

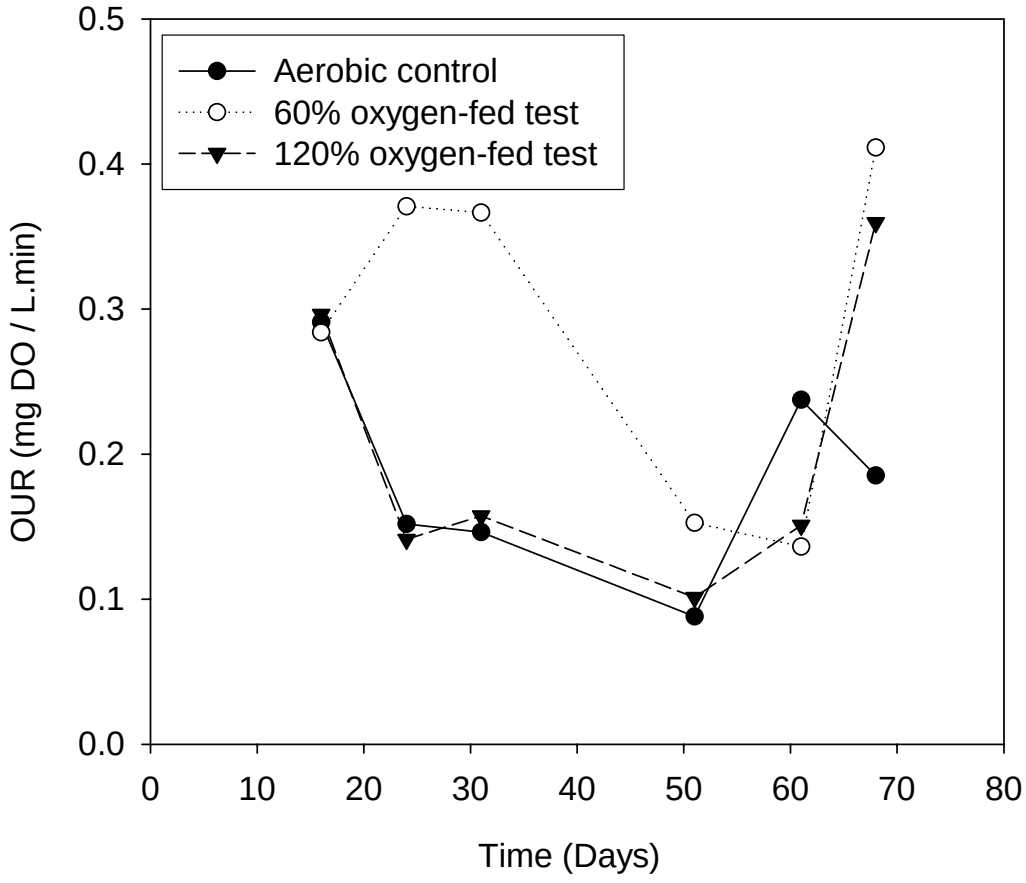


Figure 4.6. The comparison of the OUR values of developed granules in three types of reactors.

As discussed above, cultures respond to the environmental stresses by changing their physiological characteristics, behaviours, structures and surface properties (Bossier and Verstraete, 1996; Mazumder et al., 2000; Liu and Tay, 2004; Liu et al., 2004; Qin et al., 2004). This also explains the survival of aerobic cultures constituting the combined granules despite of the microaerobic and even

anaerobic conditions (Table 4.1) prevailing in the test reactors and noteworthy SOUR values. Zhu and Wilderer (2003) explained the survival of the aerobic granules exposed to one-week of anaerobic idle time by shifting to the dormant stage and lowering the enzymatic activity. Aerobic granules regained their former oxygen consumption activities in one week after the normal aerobic operating conditions were applied. Aerobic granules developed under microaerobic (Peng et al., 1999, 2001) and alternating aerobic / anoxic conditions (Jang et al., 2003) have been already demonstrated. Potter et al. (2000) stated that regulatory mechanisms enable bacteria adapt to the changes in the availability of oxygen. Bacteria encounter stress due to both excess oxygen and oxygen starvation, and have consequently developed defense mechanisms to survive both types of stress.

Tables 4.2 and 4.3 revealed that combined granules exhibited fluctuating SOUR and SMA values during 68 days of operation period. The fluctuating activities may be related with the change in the amounts of anaerobic and aerobic cultures constituting the combined granules. In other words, changes in the reactor content such as oxygen and substrate or limited oxygen and substrate diffusion into the inner parts of the granules may affect the fraction of viable aerobic over anaerobic culture population in the granules. Thus, SMA and SOUR activities might fluctuate due to the changing ratio of aerobic to anaerobic culture population in the granules throughout the granulation process. A similar phenomena was also demonstrated for aerobic granules where the fraction of nitrifying population over heterotrophic population increased with increased substrate N/COD ratio (Yang et al., 2003; Liu et al., 2003c). Because granulation is a very complex process, the detailed explanation of the granulation process with respect to the time dependent activities might be misleading.

The SCOD removal efficiencies of the test reactors were more or less the same as the control reactors (Figure 4.7). In spite of the alternating anaerobic / aerobic and/or microaerobic conditions, the similar efficiencies of the combined granules as those of anaerobic granules indicated that anaerobic cultures were well-

protected. On the other hand, similar SCOD removal efficiencies of combined granules as those of aerobic granules also revealed the survival of aerobic cultures located at the outer parts of the combined granules during anaerobic or microaerobic phases of alternating conditions.

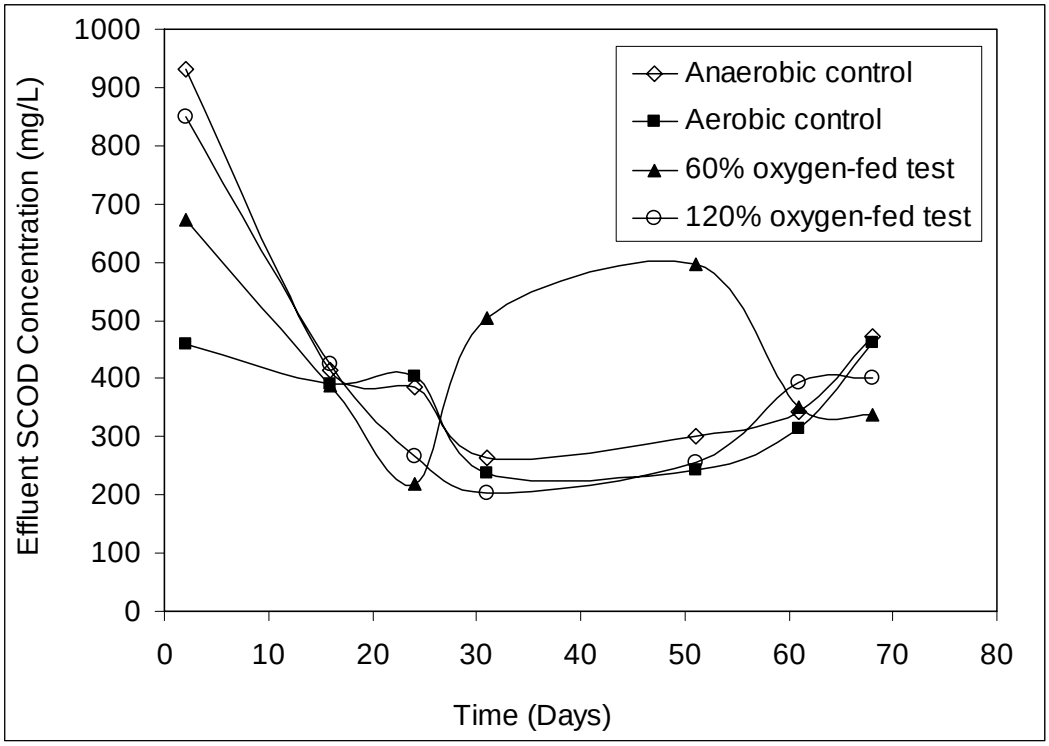


Figure 4.7. SCOD removal efficiencies of the granules in the reactors.

A similar performance of combined granules as those of controls, in fact, is not a disadvantage. On the contrary, combined granules might be superior to anaerobic or aerobic granules in terms of treatment performances. Because, in addition to similar performances, combined granules also overcame the drawbacks (Liu and Tay, 2004) of both anaerobic and aerobic granules such as the need for long start-up and low stability due to fast growth rate, respectively, under the studied experimental conditions.

4.2. USB Reactor Experiments

4.2.1. USB Reactor Study 1: Development of Combined Granules from Suspended Anaerobic and Aerobic Cultures

First USB reactor study was performed to investigate the granulation possibility of a mixture of suspended anaerobic and aerobic cultures in continuously operated USB reactors. It was also aimed to determine the aeration rate leading to alternating aerobic and/or microaerobic / anaerobic conditions in the reactors and to examine the effects of aeration strength (accordingly DO concentration) on granulation. Therefore, two identical USB reactors, namely R1 and R2, were continuously aerated with flow rates of 10 and 100 mL air/min, respectively (Figure 4.8).

Figure 4.8. Two USB reactors operated in the temperature-controlled room.

Ethanol, which led to the largest and the most stable granules in the first and second semi-continuous reactor studies (Sections 4.1.1 and 4.1.2), was used as the carbon source. Because the granulation was the main purpose, the values of the operating parameters were selected considering the granulation studies in UASB reactors and aerobic SBRs (Section 3.3.2.1). The initial influent SCOD concentration, SOLR and HRT were set as 2-3 g/L, 1.5 g COD/L.day and 1.5 day, respectively (Lettinga et al., 1984; Lettinga and Hulshoff Pol, 1986; Wu et al., 1987; Ghangrekar et al., 1996; Morgenroth et al., 1997; Beun et al., 1999; Yu et al., 2001). The performances of the USB reactors were monitored by pH, VFA, alkalinity, SCOD, MLSS and MLVSS. Furthermore, the sludge samples were analyzed for their particle sizes.

Experiments lasted only 49 days due to the sludge washout in the reactors. Only the main results that formed the base of the further USB reactor studies were discussed in this section. The experimental results were not given herein but illustrated in Appendix E.

During the operation period, conditions prevailing in the reactors were monitored by DO analyses and supernatant color. The supernatant color of R2 turned into pink from purple (the original color of BM exhibited by the resazurin dye) two days after the start-up (Figure 4.8), indicating the oxidized conditions (Zitomer, 1998). However, the supernatant of the R1 was colorless most of the time, which revealed the more reduced conditions (less than – 50 mV). DO analyses verified the conditions prevailing in the reactors, which was microaerobic / anaerobic in R1 (10 mL/min air) while aerobic in R2 (100 mL/min). DO concentrations between Days 0-22 ranged from 0.0 to 0.5 mg/L in R1 and from 3.5 to 5.0 mg/L in R2.

Because of the high turbulence (due to high aeration rate) and apparent sludge decrease in R2, the aeration rate was decreased from 100 to 60 mL/min by Day

22. After decreasing the air flow rate, DO concentration decreased to 1.1 mg/L and remained around 2.0-2.4 mg/L. Sludge loss was also observed in R1. As already mentioned, the air flow rate applied in R1 was 10 mL/min, which was not necessarily the minimum aeration rate required for granulation. However, it was the minimum flow that could be measured by the flow meter and prevented the clogging of the diffusers located at the bottom of the reactor. Therefore, the air flow rate of R1 (10 mL/min) could not be decreased further but remained constant.

As mentioned previously, experiments were discontinued around 50 days, because the loss in the MLVSS content of USB reactors was drastic. High sludge losses observed in both USB reactors might have been due to either the high aeration rates (i.e. high shear force) of long periods and in turn wash-out of the cultures or due to the granulation process. The decreasing of the MLVSS content or wash-out of the microorganisms that do not take place in the granulation process is an expected initial step and indicative of granulation start-up in UASB reactors and aerobic SBRs (Hulshoff Pol et al., 2002; Wang et al., 2004). However, in R2 the initial MLVSS concentration of almost 9000 mg/L decreased gradually to 1200 and then to 400 mg/L by the end of Day 22. Similarly, the initial MLVSS content of R1 decreased gradually from 9000 mg/L to 5300, 1700 and finally to 900 mg/L by Day 22. In other words, the sludge losses (as MLVSS) in R1 and R2 reached to 90 and 95%, respectively, by Day 22. This was mainly attributed to the high shear force as a result of high air flow rates. Usually shear force is known to have a crucial role for granulation in upflow reactors. However, high shear force might also lead to the washout of the cultures (Liu and Tay, 2004).

Despite of the decreasing MLVSS contents during the first 22 days, cultures in both reactors grew in size and had compact structures as granules. Considering the anaerobic and aerobic granulation studies (Schmidt and Ahring, 1996; Peng et al, 1999; Yu et al., 2001; Liu and Tay, 2004; Hu et al., 2005a, 2005b), granular cultures in this thesis were defined as the ones having compact and rigid structure

(unlike the floc-like pellets of loose and soft appearance) with particle size diameters above and equal to 0.1-0.2 mm (Section 4.1.1). The cultures in R2 reached to a median size of 0.14 mm and maximum size of 0.6 mm by Day 12, which was two-fold greater than the maximum sized-granules in R1. Besides, the median particle sizes of the granules in R1 reached to 0.20 mm on Day 17. Therefore, it can be said that in both USB reactors seeded with anaerobic and aerobic suspended cultures, combined granules of small sizes were developed. But it should be noted that floc-like pellets were also detected in the reactors.

In spite of the high sludge losses in both reactors, the experiments lasted one month more after Day 22 (till Day 49) to monitor the changes in the particle sizes of the cultures. It is reported in literature that to support and speed up the granulation the SOLR should be gradually increased after at least 80% COD reduction was achieved (Lettinga and Hulshoff Pol, 1986; Hulshoff Pol et al., 2002). Therefore, with the achievement of COD removal efficiencies greater than 80%, SOLRs of both reactors were increased to 3 g COD/L.day. In other words, HRTs were decreased to 1 day on Day 25. However, particle sizes of the cultures did not change significantly; thus, reactors were shutdown on Day 49.

As mentioned previously, although experiments could not be completed, the results obtained formed the base of the further USB reactor studies. The main findings of the first USB reactor study were briefly given below:

- DO concentrations measured in the USB reactors aerated with 10 and 60 mL/min were 0.0-0.5 and 1.1-2.4 mg/L, respectively. DO concentrations and supernatant color of the reactors indicated the anaerobic/microaerobic, and aerobic conditions in the 10 and 60 mL/min-aerated USB reactors, respectively.

- Throughout the operation period, the 60 mL/min-aerated USB reactor (R2) displayed greater COD removal efficiencies (70-97%) than the low aerated one (R1) (20-85%).

- A portion of suspended cultures in both reactors formed small granules. The combined granules in the 60 mL/min-aerated USB reactor displayed a faster growing trend compared to the 10 mL/min-aerated one for the first two weeks. The floc-like pellets were also observed in the reactors.

- In 34 days, granules with initial diameter of 0.03 mm grew up to 0.18 mm and 0.13 mm in the 10 and 60 mL/min-aerated USB reactors, respectively.

- The VSS contents of both USB reactors decreased gradually, leading to almost 90-95% sludge loss, with a faster trend in the 60 mL/min-aerated one. Although decrease in the MLVSS content was expected during granulation (Hulshoff Pol et al., 2002), such high sludge losses were mainly due to the continuous aeration of the reactors at high air flow rates (high shear force).

- Granulation takes approximately 2-4 months (or more) in the UASB reactors without any inert particles (Yu et al., 1999; Liu et al., 2003a; Liu and Tay, 2004). Therefore, if the excessive washout of the reactor content was prevented, it might be still possible to develop granules from suspended anaerobic and aerobic cultures in USB reactors. To this purpose, in further USB reactor experiments, the lowest aeration flow rate (10 mL/min) should be applied intermittently.

- As a solution to the sludge washout and developing combined cultures in USB reactor, suspended anaerobic seed culture could be substituted with anaerobic granular sludge. Thus, the washout of suspended aerobic cultures can be prevented by means of their attachment to the dense anaerobic granules.

4.2.2. USB Reactor Study 2: Development of Granular Combined Cultures from Suspended Anaerobic and Aerobic Cultures

The second USB reactor study was conducted to develop combined granules from a mixture of suspended anaerobic and aerobic cultures in a USB reactor. The USB reactor operated in the temperature-controlled room is illustrated in Figure 4.9.

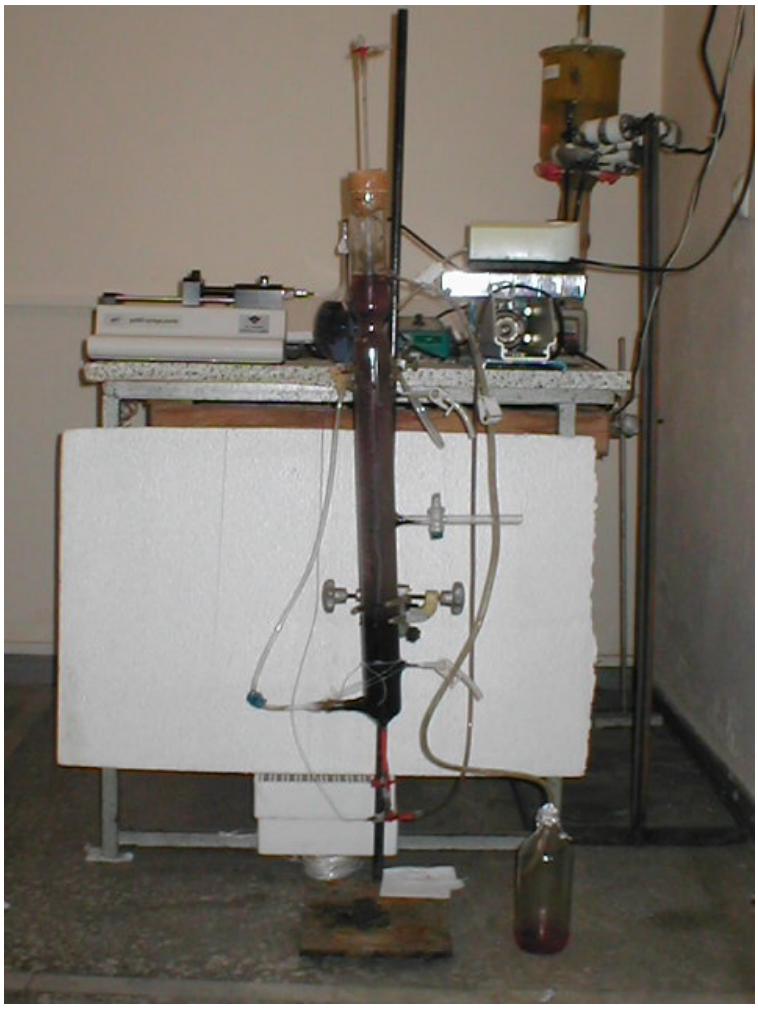


Figure 4.9. Second USB reactor set-up in the temperature-controlled room.

4.2.2.1. Performance of the USB Reactor

The USB reactor was operated for 370 days. The reactor has been aerated during 4 hours/day with an air flow rate of 10 mL/min. Such a periodic aeration was preferred to prevent sludge washout which was previously experienced due to continuous aeration in USB reactor study 1 (Section 4.2.1). Besides, periodic aeration might also supply the acclimation of seed cultures and in turn the enrichment of combined cultures by means of achieving periodic aerobic and/or microaerobic / anaerobic conditions in the system. DO analyses indicated that during the aeration period of 4 hours, DO concentration in the reactor ranged within 0.2-0.8 mg/L. With the cessation of the aeration, anaerobic conditions were maintained (DO was 0.0 mg/L). DO results revealed that during the 370 days of operation periodic anaerobic and microaerobic conditions (DO < 1 mg/L, Yerushalmi et al., 2001) were achieved in the reactor content.

During 370 days, the performance of the reactor was monitored by the effluent pH, VFA and alkalinity contents. The influent and effluent VFA, alkalinity and pH values of the reactor were tabulated in Table 4.4. Because both anaerobic and aerobic cultures were used as seed sludge and the former being more sensitive compared to the aerobic ones, optimum anaerobic operational conditions set for pH, VFA and alkalinity were taken as base for performance control and assistance of the reactor. The optimum operational conditions for anaerobic systems are specified as 6.5-8.2, below 250 mg/L and 1000-5000 mg/L for pH, VFA and alkalinity, respectively (Speece, 1996).

As seen in Table 4.4, the VFA and alkalinity values of the USB reactor were among the ranges given. Effluent pH values were above the ranges stated as 6.5-8.2 (Speece, 1996); however, pH analyses were performed for the 24-hour composite samples. The pH analyses performed for the reactor content indicated that USB reactor was operating at pH range of 7.5-8.35. It can be said that the

reactor was operating properly under the periodic anaerobic and microaerobic conditions and varying influent SCOD concentrations.

Table 4.4. The influent and effluent VFA, alkalinity and pH values of the reactor.

Parameters	Value
Influent pH	7.3-7.8
Effluent pH	8.8-9.3
Influent VFA (mg/L)	0
Effluent VFA (mg/L)	0-220
Influent Alkalinity (mg/L)	1500-2000
Effluent Alkalinity (mg/L)	1500-1900

During the operation period of 370 days, the performance of the seed cultures exposed to periodic anaerobic and microaerobic conditions was assessed by SCOD and BOD_5 removal efficiencies. The operating parameters and the results of the experiments are given in Figure 4.10. As seen in Figure 4.10, the operational period can be described in three phases: Phase 1 as the granulation start-up; Phases 2 and 3 as the low strength wastewater application with synthetic and municipal wastewater, respectively. The operational performances and/or the development of the combined cultures were explained below for each phase separately.

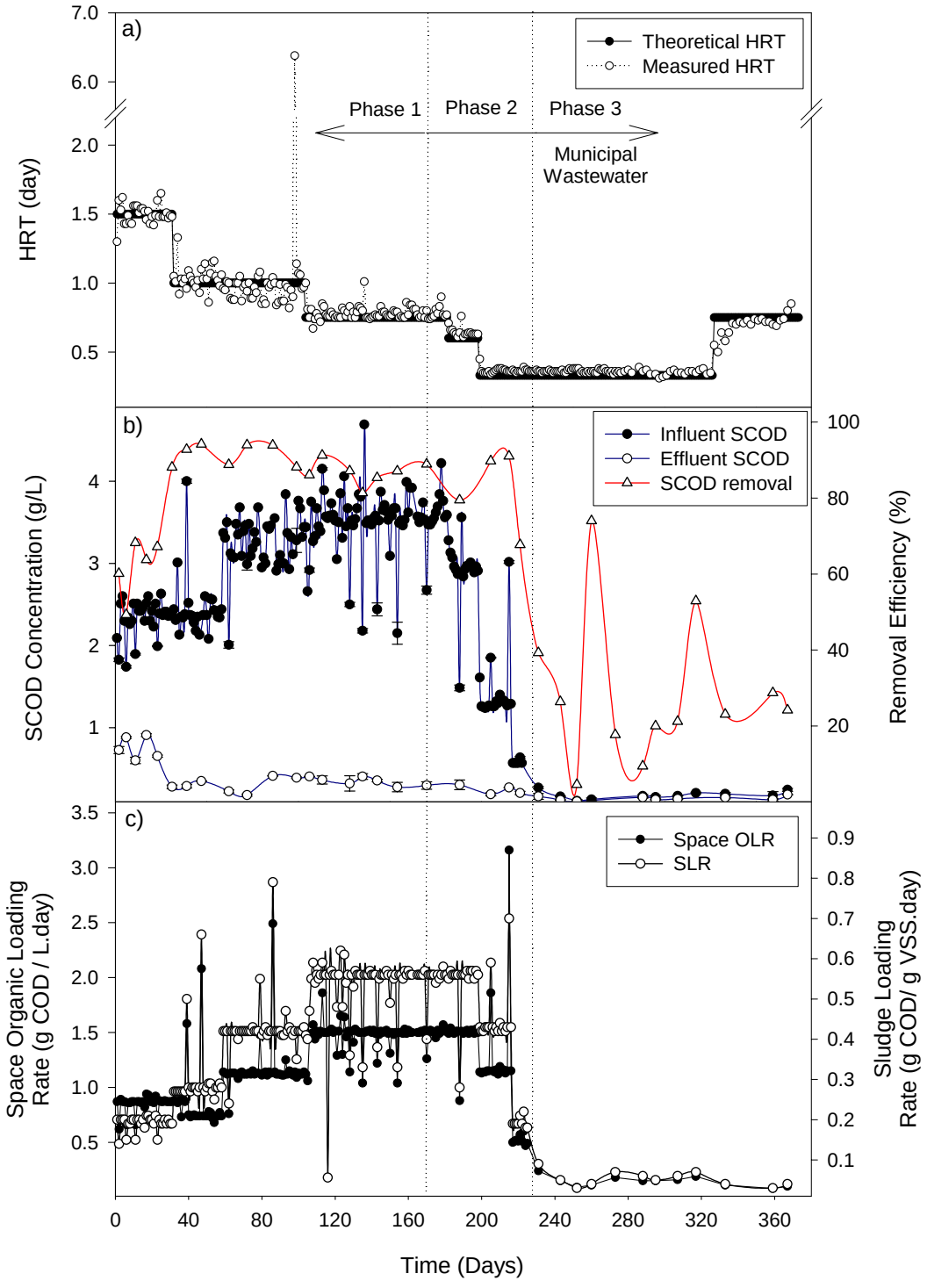


Figure 4.10. The operational conditions and results of the second USB reactor study.

Phase 1 (Granulation Start-up): The granulation start-up lasted almost 170 days, which is the expected approximate period for development of anaerobic granules up to 2 mm (Yu et al., 1999). Considering the granulation start-up parameters in UASB reactors and aerobic SBRs (Section 3.3.2.2), HRT, influent SCOD concentration and SLR were initially set as 1.5 day, 2.5 g/L and 0.2 g COD/g VSS.day, respectively. As seen in Figure 4.10b, the SCOD removal efficiency increased to 88% (from 50-60%) after 31 days of operation. It is reported in literature that to support and speed up the granulation the SLR (or SOLR) should be gradually increased after at least 80% SCOD reduction was achieved (Lettinga and Hulshoff Pol, 1986; Hulshoff Pol et al., 2002). Thus, the SLR was increased to 0.3 g COD/g VSS.day, while HRT was decreased to 1 day on Day 31. From Day 31 to Day 60, the SCOD removal efficiency was around 93-94%, thus, the influent SCOD concentration was increased to 3.5 g/L, while the SRT was increased to 0.45 g COD/g VSS.day (Figure 4.10b, c). Due to the steady state SCOD removal efficiencies (88-94%), HRT was decreased to 0.75 day on Day 104, while SRT was increased to 0.55 g COD/g VSS.day. The SCOD removal performance of the system was not negatively affected but remained around 81-91% from Day 104 to Day 170. It was observed that throughout the granulation start-up period (Phase 1), the system displayed a high performance in terms of SCOD removal efficiency. Therefore, it may be concluded that the seed cultures might have adapted to the operating conditions (influent SCOD, HRT, and 4 hours of daily aeration).

The VSS analyses performed in the reactor effluent indicated a continuous sludge washout up to 210 mg/L for the first 10 days. Effluent VSS concentration later decreased to 120 mg/L and then down to 50 mg/L till Day 130 (Figure 4.11). However, this washout was not drastic but was as expected. As mentioned previously, MLVSS content in a reactor decreases due to the washout of the microorganisms not participating in the granulation process, which indicates the granulation start-up in UASB reactors and aerobic SBRs (Hulshoff Pol et al., 2002; Wang et al., 2004). After Day 130, the amount of effluent sludge remained

below 50 mg/L. From Day 230 to the end of the experiment (during Phase 3), the effluent VSS concentration was almost below 10 mg/L. The initial VSS content of the reactor (3.5 g) reached to 2.0 g by the end of the experiment.

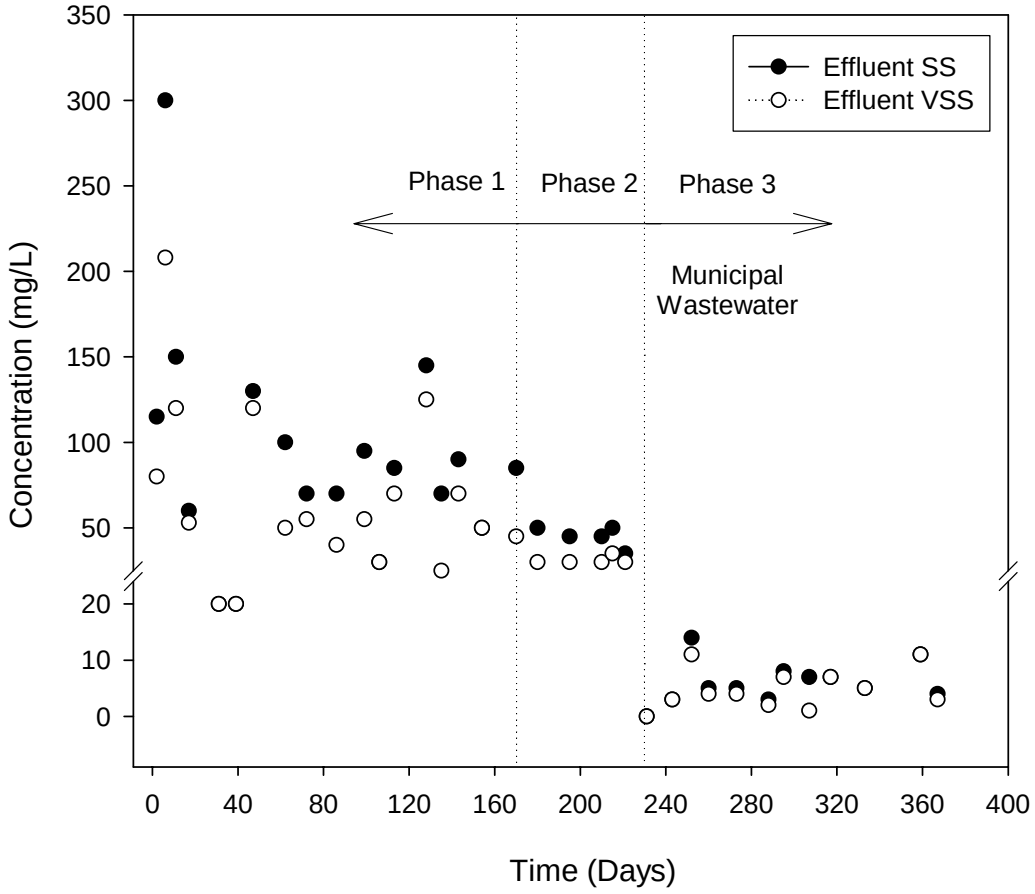


Figure 4.11. The effluent SS and VSS concentrations of the USB reactor.

To monitor granulation process, sludge samples periodically withdrawn from the reactor were analyzed for particle size determination / image analyses. The images of the sludge samples taken on varied days are illustrated in Figure 4.12.

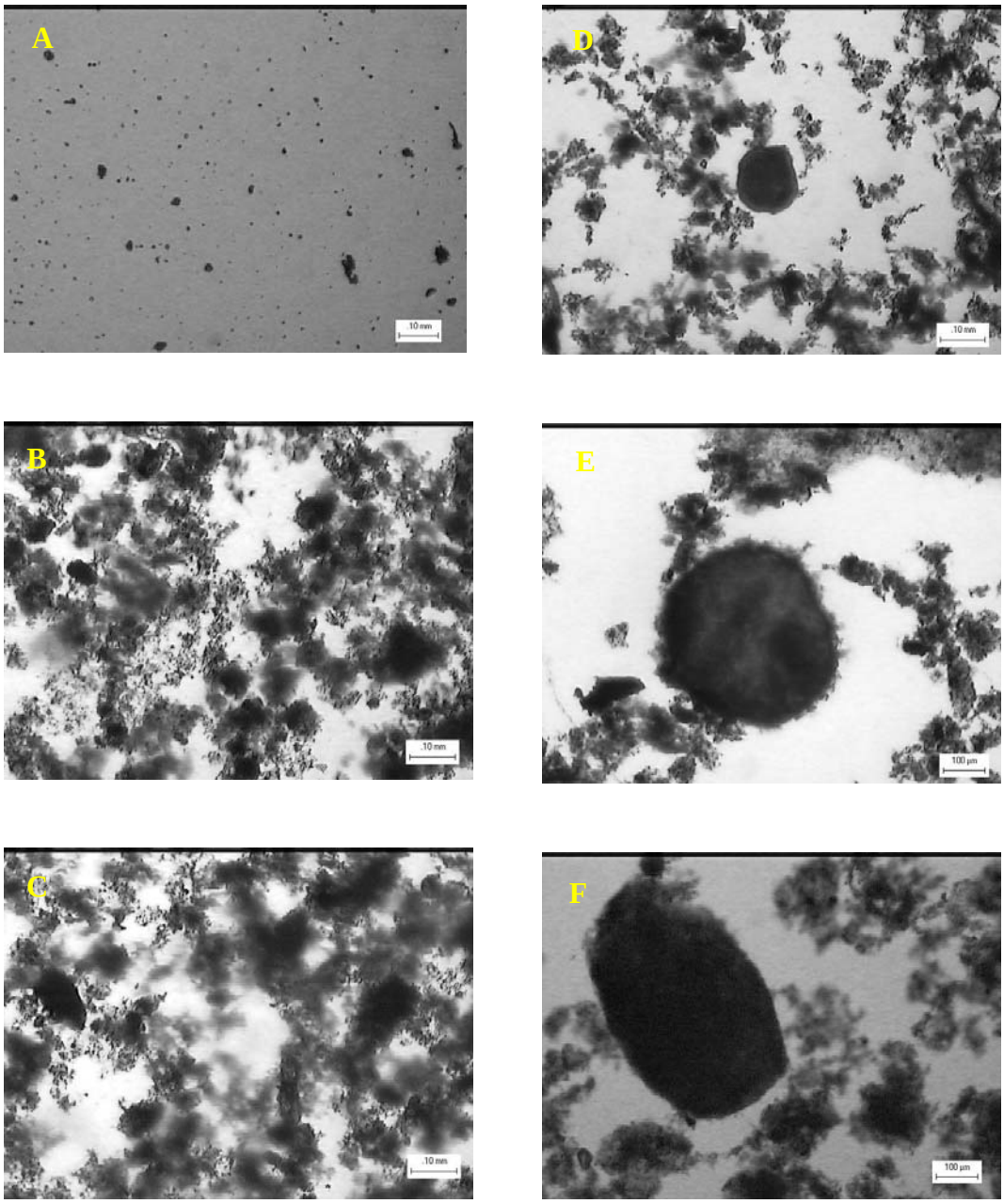


Figure 4.12. The images of the sludge samples taken on a) Day 46, b) Day 90, c) Day 116, d) Day 148, e) Day 327, f) Day 370. (Bar = 0.1 mm)

During Phase 1, it was observed that suspended cultures (Figure 4.12a) changed to floc-like sludge / pellets. Floc-like pellets can be seen in Figure 4.12b, c, d, e and f around the granular cultures. Almost all reactor content turned into floc-like pellets, which could easily be noticed during settling of the sludge bed after cessation of the aeration. In time, a portion of floc-like pellets gradually developed to compact granules with rigid structure (Figure 4.12d, e and f). Towards the end of the experiment (Day 370), the ratio of granular cultures with respect to floc-like pellets increased. However, floc-like pellets still existed in the reactor content till Day 370.

Development of combined granules was monitored through Figure 4.12a to 4.12f. Image analyses indicated that seed cultures of 20-30 μm (Figure 4.12a) slightly grew to a size of 50-80 μm, 0.1-0.13 mm and 0.14-0.25 mm by Days 90, 116 and 148, respectively (Figure 4.12b, c, d). Besides, the floc-like structure of the pellets (as in Figure 4.12b and c; Days 90, 116) turned into a rigid form (Figure 4.12d) by Day 148. The rigid combined granules grew in size till the end of the experiment which indicates their acclimation to the operating conditions. The images of the combined granules with sizes of 0.375 and 0.5 mm can be seen in Figure 4.12e and f, respectively. Particle size analyses indicated that combined granules with rigid and compact structures reached to a median size of 0.8 mm (with a range of 0.4-1.92 mm) by the end of the experiment.

Literature indicates that both aerobic and anaerobic granules are developed in shorter periods compared to this study. Yu et al. (2001) reported that 90% of the cultures grown in a UASB reactor for 30 days were lower than 0.2 mm. Besides, in a sludge granulation study 10% of the granules were stated to grow to a size of 2 mm in 95 days (Yu et al., 1999). The period for aerobic granulation was reported as 2-4 weeks (Tay et al., 2004a, 2004b; Qin et al., 2004). Aerobic granules of 0.35 mm were reported to develop in one week under aerobic conditions (Qin et al., 2004). On the other hand, in an SBR study performed under microaerobic conditions (DO: 0.7-1.0 mg/L), aerobic granules with similar sizes

(0.5 mm) were developed after one month of operation (Peng et al., 2001). In this study cultures of 20-30 μm slightly grew to a maximum size of 0.25 mm in 148 days. It should be noted that this study is the first in literature to develop granules from a mixture of suspended anaerobic and aerobic seed cultures under alternating anaerobic / microaerobic conditions. The prevailing conditions were not either anaerobic or aerobic, but periodical anaerobic and microaerobic. Therefore, such a slowly proceeding granulation process and in turn small granules might be attributed to the mixed anaerobic and aerobic seed cultures and alternating anaerobic / microaerobic conditions (DO of 0.0 mg/L to 0.2-0.8 mg/L) achieved in the reactor content.

Anaerobic granulation studies (in UASB reactors) indicate the liquid upflow velocity and gradually decreased HRT (i.e. hydrodynamic shear force and selective pressure) or gradually increased OLR / SLR as the significant factors leading to granulation. Aerobic granulation studies mostly performed in SBRs also point out the superficial upflow air velocity (shear force), and short HRT and settling time (selective pressure) as the important factors of granulation. However, in this study, the liquid upflow velocity achieved in the reactor was 0.01-0.02 m/hr, which was lower than the ranges required for anaerobic granulation as 0.03-0.04 m/hr (Speece, 1996) to 0.5 m/hr (Lettinga and Hulshoff Pol, 1986). The superficial upflow air velocity (0.01 cm/s) was also much lower than the minimum threshold value required for aerobic granulation in SBRs (1.2 cm/s, Tay et al., 2001a). This considerable difference between the studied superficial upflow air velocity and those of SBR studies should not be considered suspiciously. SBRs are operated in discontinuous mode; therefore the sludge loss only occurs during the settling period of the cyclic operation as a selection pressure for granulation. However, in this study, due to continuous operation both air flow and liquid flow might lead to hydraulically supported sludge washout. Thus, in order to prevent hydraulically supported sludge washout (as observed in the first USB reactor study), minimum air flow that could be measured by flow meter and in turn minimum superficial upflow air velocity was applied (for four hours). As a result,

it can be concluded that granulation process observed in USB reactor might not be related with the superficial upflow liquid and air velocities.

As mentioned previously, granulation start-up parameters of USB reactor such as SOLR, influent SCOD, HRT, SLR and their applications were well within the ranges of the parameters set for anaerobic and aerobic granulation in UASB reactors and SBRs, respectively (Figure 4.10 and Table 2.2, Section 2.2.1). Therefore, these parameters might have affected the granulation in USB reactor. In the first USB reactor study, the granules with median sizes of 0.18 mm were developed in almost one month (34 days) in the USB reactor (R1) aerated with 10 mL/min (Section 4.2.1). However, in this study, the granules of 0.1-0.25 mm were developed in 4 to 5 months. As seen in Figure E.2 (in Appendix E) and Figure 4.10, the operating parameters of both reactors were same for the first 30 days except SOLR. SOLR value of R1 (Figure E.2) was almost two-fold greater than that of USB reactor used in this section. High SOLR might result in increased granule sizes (Quarmby and Forster, 1995; Tay et al., 2001a, 2001b; Liu and Tay, 2004). In addition, R1 was aerated with 10 mL air/min for 24 hours, while the aeration period in this study was only 4 hours. Longer aeration period means shear force of long duration and also greater amount of oxygen (that supports cultivation of greater amount of aerobic cultures). These might explain the difference of the sizes of granules developed in R1 and in this section.

The aeration strength and period (i.e. the oxygen amount) and the resultant alternating cyclic conditions might have an important role in granulation start-up of suspended mixed anaerobic and aerobic cultures in USB reactors and its duration. As the microaerobic conditions are prevailing, filamentous bacteria might be developed at low DO level, which favors the flocculation (Bossier and Verstraete, 1996). Thereafter, with the formation of loose floc-like pellets, anaerobic cultures might locate inside these pellets for protection against microaerobic conditions as a survival mechanism. However, it should be noted that not all of the pellets changed into compact granules, but remained in floc-like

structure till the end of the experiment (Figure 4.12f). This might be attributed to the insufficient oxygen amount. In other words, low aeration strength (10 mL/min), short aeration period (of four hours) and lower DO might affect the amount of aerobic cultures (especially the floc formers favored at high DO and producing ECP). Because of lower DO, ECP production might not be favored. ECPs (especially extracellular polysaccharides), which are highly hydrated gel acting as a cementing substance and produced at high DO (De Beer et al., 1996; Peng et al., 2001), play an important part in maintaining the formation and structural integrity of the granules (Tay et al., 2001c). Therefore, due to inadequate oxygen amount, low DO concentrations and ECPs, not all of the floc-like pellets developed into granular cultures. The portion of the pellets developing into granules of compact structure might be due to the slowly-growing anaerobic cultures inside the pellets. In other words, it might be the anaerobic cultures that mostly determined the formation of compact granules, which explains the slowly proceeding granulation period (4-5 months). To speed up the granulation process via aerobic cultures and convert all floc-like pellets into granular cultures, alternating cyclic aerobic to anaerobic conditions of adequate periods rather than microaerobic to anaerobic conditions should prevail in the reactor. To this purpose, the reactor should be aerated with a better diffuser system leading to improved oxygen transfer efficiency and/or with a greater air flow rate in (adequate) periods. But in order to prevent washout of the cultures at greater flow rates (as observed in USB reactor study 1), the reactor can be operated in a discontinuous mode during the aeration period as in SBRs, or a better gas-solid separator should be used.

As a result, for optimum combined granulation (involving all cultures and in short period) periodic high DO and in turn alternating cyclic aerobic / anaerobic (or microaerobic) conditions are required. This also explains the portion of floc-like pellets observed in USB reactors, namely R1 (microaerobic conditions) and R2 (aerobic conditions) operated in USB study 1 (Section 4.2.1). Cyclic aerobic to anaerobic (or microaerobic) conditions of adequate periods might provide

achievement of balance among the different culture types constituting the granules. Results are also consistent with the results of semi-continuous reactor study 4, where combined granules were developed under alternating cyclic aerobic to anaerobic (60% oxygen-fed tests) and aerobic to microaerobic conditions (120% oxygen-fed test) of adequate periods (Section 4.1.4.1).

In spite of the slow granulation, the cultures exposed to influent SCOD of 3.5 g/L at an HRT of 0.75 day displayed SCOD removal efficiencies of 81-91%. The combined granules/cultures that were developed under the alternating anaerobic / microaerobic conditions had significant removal efficiencies as well as SMA / SOUR activities (Section 4.2.2.2) were developed. So, the further steps (Phases 2 and 3), where the objective was the low strength wastewater treatment, were started. This does not mean that granulation was completed within 170 days (Phase 1) as granules with median sizes of 0.8 mm were developed by the end of the experiment. However, due to achievement of combined cultures that survived and operated well under alternating conditions, Phase 2 was started.

Phase 2 (Low Strength Wastewater Application –synthetic wastewater-): In Phase 2 (Days 171-225), the strength of the synthetic wastewater (prepared with ethanol and BM as in Phase 1) was gradually decreased from 3.5 g/L to 3 and then to 1.25 g/L SCOD to acclimate the combined cultures to low strength wastewater (Figure 4.10b). Besides, HRT was gradually decreased from 0.75 day to 0.6 and 0.33 day. When the SCOD and HRT values were 1.25 g/L and 0.33 days, respectively, SCOD removal efficiencies of 80-91% were obtained. Thus, the influent SCOD was decreased to 0.57 g/L. However, the performances of the combined cultures decreased down to 68% at that influent SCOD value and HRT of 0.33 day (Figure 4.10a, b). The strength of the wastewater was not decreased further and Phase 3 was started.

Phase 3 (Low Strength Wastewater Application -municipal wastewater-): From Day 226 till the end of the experimental period (Day 370), municipal

wastewater was fed to the reactor. The municipal wastewater had influent SCOD and BOD_5 concentrations of 155-244 mg/L and 53-118 mg/L, respectively. When the reactor was operated at an HRT of 0.33 day (8 hours), the combined granules/cultures displayed low SCOD and BOD_5 removal efficiencies of 10-53% and 15-56%, respectively. Thus, HRT of the system was increased to 0.75 day (18 hours); however, SCOD removal efficiencies did not change significantly (23-29%) (Figure 4.10a, b). Typical domestic wastewaters have a COD/BOD_5 ratio of 1.14 (Tchobanoglous and Burton, 1991). However, the municipal wastewater used in this study had COD/BOD_5 ratio of 1.43-2.92 with an average of 2.10. Therefore the low SCOD removal performances of the combined granules/cultures might be attributed to the low biodegradability of the municipal wastewater fed to the reactor.

In spite of the low SCOD performances, BOD_5 removal efficiencies increased to 66-68% and stabilized with the HRT increase from 0.33 to 0.75 day. Therefore, it was concluded that the developed combined granules/cultures had significant removal efficiencies in terms of BOD_5 for municipal wastewaters. BOD_5 removal efficiency of 66-68% is lower than that of activated sludge systems which are reported as 85-95% (Tchobanoglous and Burton, 1991). However, the effluent BOD_5 values of the USB reactor were 31-36 mg/L, which are below the effluent discharge standards of Turkey set as 45 mg/L for 24-hr composite samples of treated domestic wastewaters (SKKY, 1998). Effluent BOD_5 values were slightly greater than the European Union effluent discharge standards set as 25 mg/L (EU, 1991).

As mentioned previously, granulation process was not limited to the first 170 days (Phase 1), but slightly continued till the end of the experiment where granules with median sizes of 0.8 mm were developed. In spite of the slow rate, the continuous growth observed from Day 170 to 370 also indicated that periodical anaerobic / microaerobic conditions and low strength wastewater did not negatively affect the granulation process.

4.2.2.2. Microbial Activity and Physical Characteristics

Microbial Activity: In order to determine the activities of the combined granules/cultures exposed to 10 mL/min air flow for 4 hours/day, SMA and SOUR analyses were performed. The results of the analyses are given in Table 4.5. Because the reactor was operated at varying influent SCOD, SOLR, SLR and HRT values, SMA and SOUR values were not evaluated with respect to time.

Table 4.5. The results of the SMA and SOUR analyses.

Phases	Days	SMA (mL CH_4/g VSS.hr)	SOUR (mg DO/g VSS.hr)	Inf. SCOD (mg/L)	HRT* (days)
Phase 1 (Granulation start-up with EtOH)	46	33.04	14.76 ± 1.25	2500	1 (24)
	98	27.16	9.72 ± 0.15	3200	1 (24)
	155	44.99	15.62 ± 0.13	2000	0.75 (18)
Phase 2 (Synthetic WW with EtOH)	219	59.30	18.50 ± 0.04	600	0.33 (8)
Phase 3 (Municipal WW)	265	76.89	49.81 ± 0.20	130	0.75 (18)
	327	44.80	74.46± 0.51	200	0.75 (18)
	370	11.44	25.86 ± 0.07	250	0.75 (18)

* Values given in parentheses are in terms of hour.
Inf: influent; EtOH: ethanol; WW: wastewater.

As seen in Table 4.5, the combined granules/cultures developed in USB reactor under alternating cyclic anaerobic and microaerobic conditions had SMA values

ranging from almost 11 to 77 mL CH_4/g VSS.hr. These values (especially the ones in Phases 1 and 2) were comparable to the values reported in literature as 16.1-57.5 mL CH_4/g VSS.hr for anaerobic granules grown on ethanol (Dolfing and Mulder, 1985; Grotenhuis et al., 1991). SMA results clearly indicated the significant amount of methane production by combined granules/cultures and in turn the existence and survival of the methanogens.

During Phases 1 and 2, SOUR values of the combined granules/cultures were in the range of 10-18.5 mg DO/g VSS.hr, which were comparable to the values given for the activated sludge as 10-19.2 mg DO/g VSS.hr (Lens et al., 1995; Zitomer and Shrout, 2000). During Phase 3, SOUR values increased to a range of 25-75 mg DO/g VSS.hr that were comparable to those of the aerobic granules reported by Tay et al. (2004a) as 29-90 mg DO/g VSS.hr. Similar SOUR activities as the aerobic cultures of activated sludge systems during the first two Phases and as the aerobic granules during Phase 3 might lead to this speculation: the ratio of aerobic to anaerobic cultures per g VSS might have increased with the acclimation of the cultures to the operating conditions. However, to decide on the processes that aerobic cultures were through by evaluating the changes in the SOUR values with respect to time might be misleading because; 1) SOUR values were obtained at varied operational conditions, 2) granulation is a very complicated process and SOUR activities of the cultures might change depending on the environmental stresses such as low DO and substrate limitation. Detailed microscopic observation therefore was required. However, the significant SOUR activities clearly indicated the existence of aerobic cultures in the combined granules/cultures.

Physical Characteristics: The physical characteristics of the combined granules developed at the end of 370 days are given in Table 4.6. Image analyses indicated that black rigid granules with clear outline and median sizes of 0.8 mm (range of 0.4-1.92 mm) were developed by the end of the experimental period. These values were comparable to the particle size ranges of the anaerobic granules and aerobic

granules reported in literature as 0.l4-5.0 mm (Schmidt and Ahring, 1996) and 0.2-5.0 mm (Liu and Tay, 2004), respectively (Table 4.6). The minimum sized-combined granules (0.4 mm) were also comparable to the aerobic granules of 0.3-0.5 mm developed in SBR under microaerobic conditions (Peng et al., 1999, 2001).

Table 4.6. Comparison of the physical characteristics of the combined granules.

Parameter	This study	Anaerobic granules		Aerobic granules		Granules at microaerobic conditions	
		Range	Ref. *	Range	Ref. *	Range	Ref. *
Particle size (mm)	0.4-1.92	0.14-5.0	1	1.0-3.0	2, 3, 4	0.3-0.5	6, 12
				0.2-5.0	5, 13		
Settling velocity (m/hr)	25	20-50	1	22-77	5, 13	-	-
		30-94	7	30-40	2, 8		
SVI (mL/g)	96	35-45	9	24-112	10	80-100	6
		18	11	50-85	8		

* Reference: 1) Schmidt and Ahring, 1996; 2) Morgenroth et al., 1997; 3) Beun et al., 2002; 4) Etterer and Wilderer, 2001; 5) Hu et al., 2005a; 6) Peng et al., 1999; 7) Batstone and Keller, 2001; 8) Tay et al., 2001a; 9) Noyola and Moreno, 1994; 10) Tay et al., 2004a; 11) Shen and Guiot, 1996; 12) Peng et al., 2001; 13) Liu and Tay, 2004.

The settling velocities of the developed combined granules were in the range of 12-60 m/hr with an average of 25 ± 12 m/hr. That wide range points out the granules of varied sizes. Nevertheless, the settling velocities of the combined granules were comparable to that of both anaerobic and aerobic granules reported in literature (Table 4.6).

The suspended anaerobic and aerobic seed cultures had initial SVI values of 210 and 280 mL/g, respectively. After 370 days of operation under alternating cyclic anaerobic / microaerobic conditions, SVI decreased to 96 mL/g. This was related to the granulation process through which the settleability of the sludge improved significantly (Noyola and Moreno, 1994; Tay et al., 2001a; Tay et al., 2004a). SVI values of combined granules/cultures were lower than the values of anaerobic granules but comparable to those of aerobic granules (Table 4.6). However, when the peculiarity of the operating conditions were considered, it was observed that SVI of 96 mL/g was well comparable to SVI of 80-100 mL/g measured for the granules (0.3-0.5 mm) developed from aerobic suspended sludge under microaerobic conditions (Peng et al., 1999). The existence of floc-like pellets in addition to granular cultures also explains the lower SVI values compared to those of anaerobic granules (Table 4.6).

The analyses performed to determine the microbial activity and physical characteristics verified that combined granules/cultures were developed from a mixture of suspended anaerobic and aerobic seed cultures. Developed granules resembled the anaerobic and aerobic granules developed in UASB and SBRs, respectively, in terms of both their activities (SMA and SOUR) and physical characteristics (structures, settling velocity and particle diameter). Thus, it was concluded that combined granules/cultures that survived and even operated well under alternating cyclic anaerobic / microaerobic conditions were developed and composed of both anaerobic and aerobic cultures.

4.2.3. USB Reactor Study 3: Development of Combined Cultures from Granular Anaerobic and Suspended Aerobic Cultures

The third USB reactor study was carried out to develop combined cultures in USB reactors for the treatment of low strength wastewaters under alternating aerobic

and/or microaerobic / anaerobic conditions. In other words, it was aimed to develop combined cultures that can survive and operate under alternating cyclic aerobic and/or microaerobic / anaerobic conditions. First USB reactor study (Section 4.2.1) revealed that even aeration at the lowest air flow rate of 10 mL/min (the minimum that could be measured by the flow meter) resulted in the washout of mixed suspended seed cultures. Therefore, in this section, suspended anaerobic seed cultures were substituted with anaerobic granular sludge to prevent sludge washout while developing combined cultures in USB reactors operating at same air flow. Thus, the washout of suspended aerobic cultures could be prevented by means of their attachment to the dense anaerobic granules.

Alternating conditions were achieved by periodic aeration of the reactors, which was another approach to prevent sludge washout. It was also aimed to investigate the effect of aeration period (thus DO content) on combined culture development and low strength wastewater treatment. Thus, all test reactors (R2, R3 and R4) were aerated with an air flow rate of 10 mL/min but with different aeration periods: R2 was aerated 4 hours/day; R3 was continuously aerated every other day; R4 was continuously aerated throughout the operation period. Aeration was not applied to R1 which was used as anaerobic control reactor and seeded only with anaerobic granular cultures. The test reactors were seeded with a mixture of anaerobic granular and suspended aerobic cultures. The set-up of the USB reactors in the temperature-controlled room is illustrated in Figure 4.13.

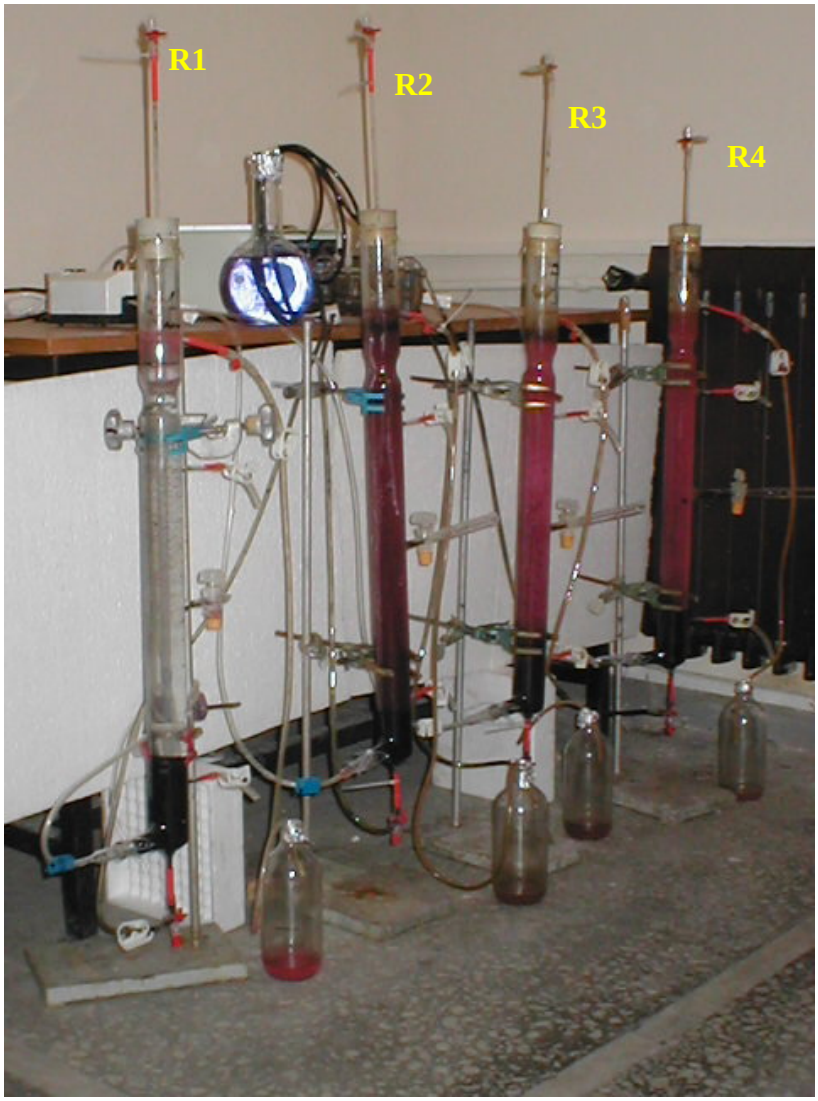


Figure 4.13. The set-up of the USB reactors conducted for study 3.

4.2.3.1. Performances of the USB Reactors

USB reactor experiments lasted 370 days. To investigate the effect of aeration period on the reactors and the conditions prevailing in each reactor, DO analyses were periodically performed. Table 4.7 shows the DO concentrations achieved in the reactors during aeration and no-aeration periods. As seen in Table 4.7, anaerobic conditions were achieved in R1 and in R2 and R3 during no-aeration

periods (DO: 0.0 mg/L). The mentioned anaerobic conditions were also verified by the supernatant color of the reactors. Resazurin, which was fed with the influent, exhibits no color at an oxidation-reduction potential relative to a standard hydrogen electrode (Eh) below approximately -50 millivolts (mV), but is pink under more oxidized conditions (Zitomer, 1998). Under anaerobic conditions, the supernatants of the R1, R2 and R3 were colorless most of the time.

Table 4.7. DO concentrations in the USB reactors measured during anaerobic and aerobic cycles.

Reactor type	DO (mg / L)	
	No-aeration period	Aeration period
R1, Anaerobic Control (no aeration)	0.0	-
R2 (4 hrs of aeration/day)	0.0	0.2-1.5
R3 (one day aerobic -24 hrs of aeration-, one day anaerobic)	0.0	0.6-2.3
R4 (24 hrs of continuous aeration)	-	0.8-5.1

As seen in Table 4.7, microaerobic / aerobic conditions prevailed in R4 most of the time and in R2 and R3 during the aeration periods. The aerobic conditions were also verified by the pink color of the supernatants of the R2, R3 and R4 (Figure 4.13). The results of DO analyses indicated that required alternating cyclic anaerobic / aerobic and/or microaerobic conditions were achieved in R2 and R3 while, microaerobic / aerobic conditions were obtained in R4 via the varied aeration protocols. However, it should be noted that anaerobic conditions were also achieved in R4 through the anaerobic granules, where oxygen could not diffuse (Kurosawa and Tanaka, 1990; Lens et al., 1995).

It was observed throughout the operation period of 370 days that all reactors operated properly in terms of effluent VFA and alkalinity contents (Table 4.8). The assessment was made considering the optimum operational conditions given for more sensitive anaerobic cultures as 6.5-8.2, below 250 mg/L and 1000-5000 mg/L for pH, VFA and alkalinity, respectively (Speece, 1996). The effluent pH values were above the pH values given for optimum anaerobic conditions as 6.5-8.2 (Speece, 1996). However, it should be noted that the reactor contents had pH values ranging from 7.25 to 7.49, which were well within the optimum pH values set for both anaerobic and aerobic treatment systems (Tchobanoglous and Burton, 1991).

Table 4.8. The influent and effluent pH, VFA, alkalinity contents of the reactors.

Parameter (mg/L)	R1	R2	R3	R4
Influent pH	6.9-7.9	6.9-7.9	6.9-7.9	6.9-7.9
Effluent pH	8.4 -9.3	8.6-9.3	8.9-9.4	9.0-9.5
Influent VFA	200-360	200-360	200-360	200-360
Effluent VFA	0-90	0-110	0-120	0-330
Influent Alkalinity	1300-1950	1300-1950	1300-1950	1300-1950
Effluent Alkalinity	1450-1750	1550-1850	1500-1800	1500-1800

The performances of the reactors were also investigated in terms of effluent VSS concentrations. The results of the SS / VSS analyses are illustrated in Figure 4.14.

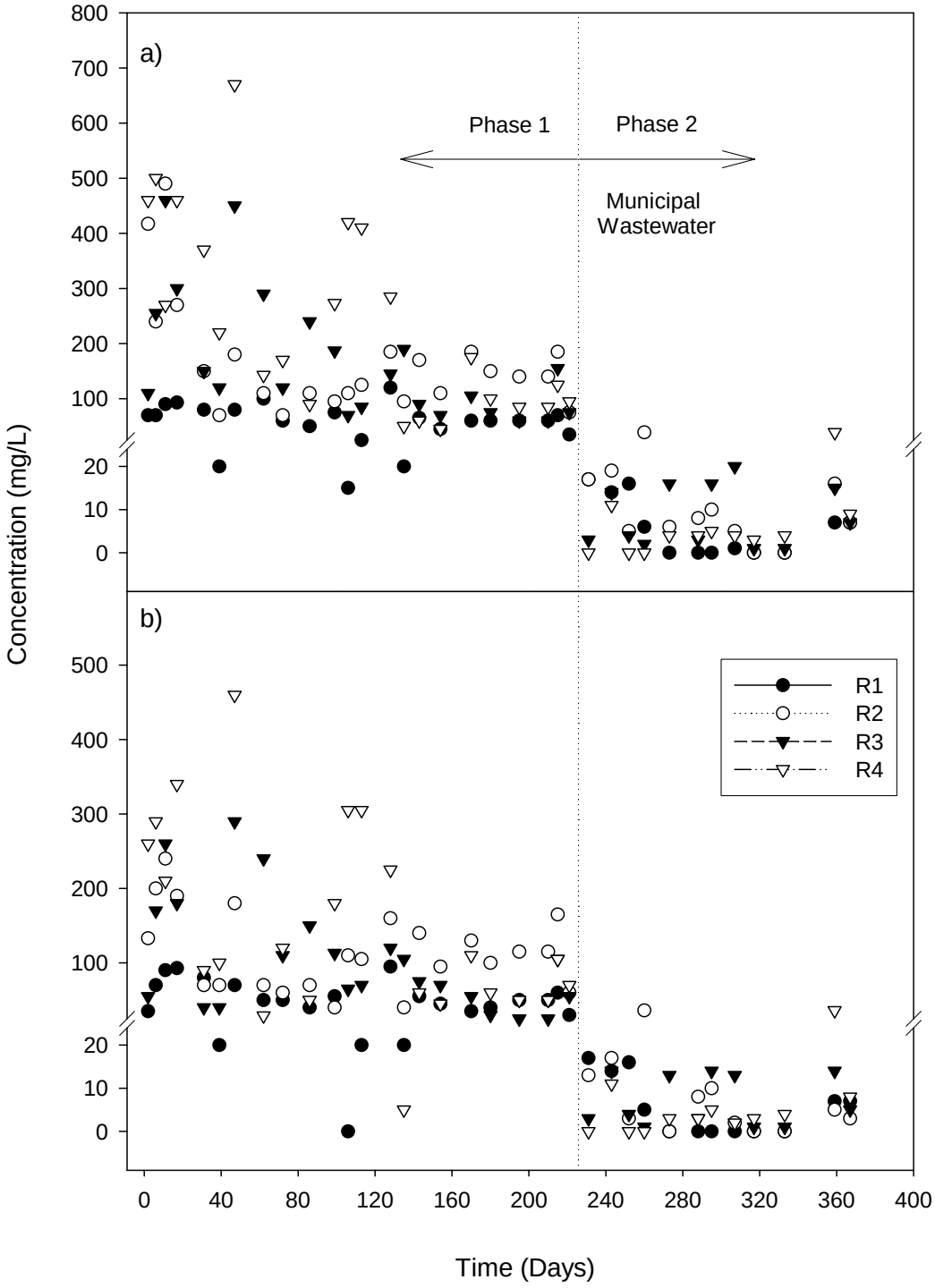


Figure 4.14. The effluent a) SS, and b) VSS concentrations of the reactors.

As seen in Figure 4.14, throughout the operation period the USB reactors R1, R2, R3 and R4 displayed maximum effluent VSS concentrations of 90, 240, 260 and 460 mg/L, respectively. The maximum effluent VSS concentrations in the test reactors increased with the increase in the aeration period as expected. In other words, as the aeration period increased from R2 to R4, the wasted sludge amount increased due to the extended duration of the shear force applied. However, the sludge washout in all test reactors was not significant enough to negatively affect their performances which are discussed in the forthcoming sections. Gas-solid separators played an important role to prevent a significant sludge loss due to aeration. The use of a gas-solid separation system in the upper portion of a USB reactor is claimed to be an essential feature, regardless of the settlement characteristics of the sludge (Liu and Liptak, 2000).

It was also observed in Figure 4.14 that the effluent VSS concentrations of all test reactors displayed a decreasing trend with respect to time. This might be due to the gradually decreasing influent SCOD concentration (from 2000 to 155 mg/L) leading to reduced new cell formation. The sudden decrease in the effluent VSS concentration observed during municipal wastewater application (Phase 2), where the effluent VSS concentrations of all reactors decreased suddenly below 20 mg/L (Figure 4.14), might be also related with low influent SCOD concentration. On the other hand, the sudden decrease might be explained by starvation-induced response of the cultures since, under starvation conditions, bacteria become more hydrophobic which facilitates microbial adhesion and aggregation (Bossier and Verstraete, 1996; Tay et al., 2001a). It is likely that microorganisms are able to change their surface characteristics when they face starvation and aggregation is their strategy against starvation (Tay et al., 2001a; Liu and Tay, 2004). Liu et al. (2003b) reported that cell surface hydrophobicity seemed not to be sensitive to the changes in the organic concentrations in the range of 500 to 3000 mg COD/L. Therefore, starvation of the cultures might not be the case in Phase 1 where SCOD concentrations ranged within 500-2000 mg/L. However, in Phase 2 the influent SCOD was as low as 155 mg/L. Therefore, the suspended cultures in the

reactor content might have increased their surface hydrophobicity due to starvation and attached on the granular cultures. This statement might also be an explanation of the sudden decrease in the effluent VSS concentrations of all reactors.

As mentioned previously, it was aimed to investigate the applicability of combined cultures in low strength wastewater treatment. Therefore, reactor performances were assessed by comparing the removal efficiencies of the reactors. The operational conditions and the results of the experiments conducted for R1, R2, R3 and R4 are illustrated in Figures 4.15, 4.16, 4.17 and 4.18, respectively. As seen in all these figures, the operational period can be described in two phases: Phase 1 as the synthetic wastewater application while Phase 2 as the municipal wastewater application. The operational results of each phase were discussed below separately.

Phase 1 (Low Strength Wastewater Application -synthetic wastewater-): Synthetic wastewater (glucose+HAc dissolved in BM solution) was fed to all reactors for 225 days. Resembling the low strength wastewaters, the SCOD value of the synthetic wastewater was initially set to 1800-2000 mg/L (Lettinga and Hulshoff Pol, 1991). The HRTs of the reactors were initially set as 1.5 days and remained constant up to Day 146. The strength of the wastewater was gradually decreased down to 500 mg SCOD/L. The next SCOD level was not adopted until at least 80% SCOD reduction was achieved (Figures 4.15, 4.16, 4.17 and 4.18, and Table 4.9).

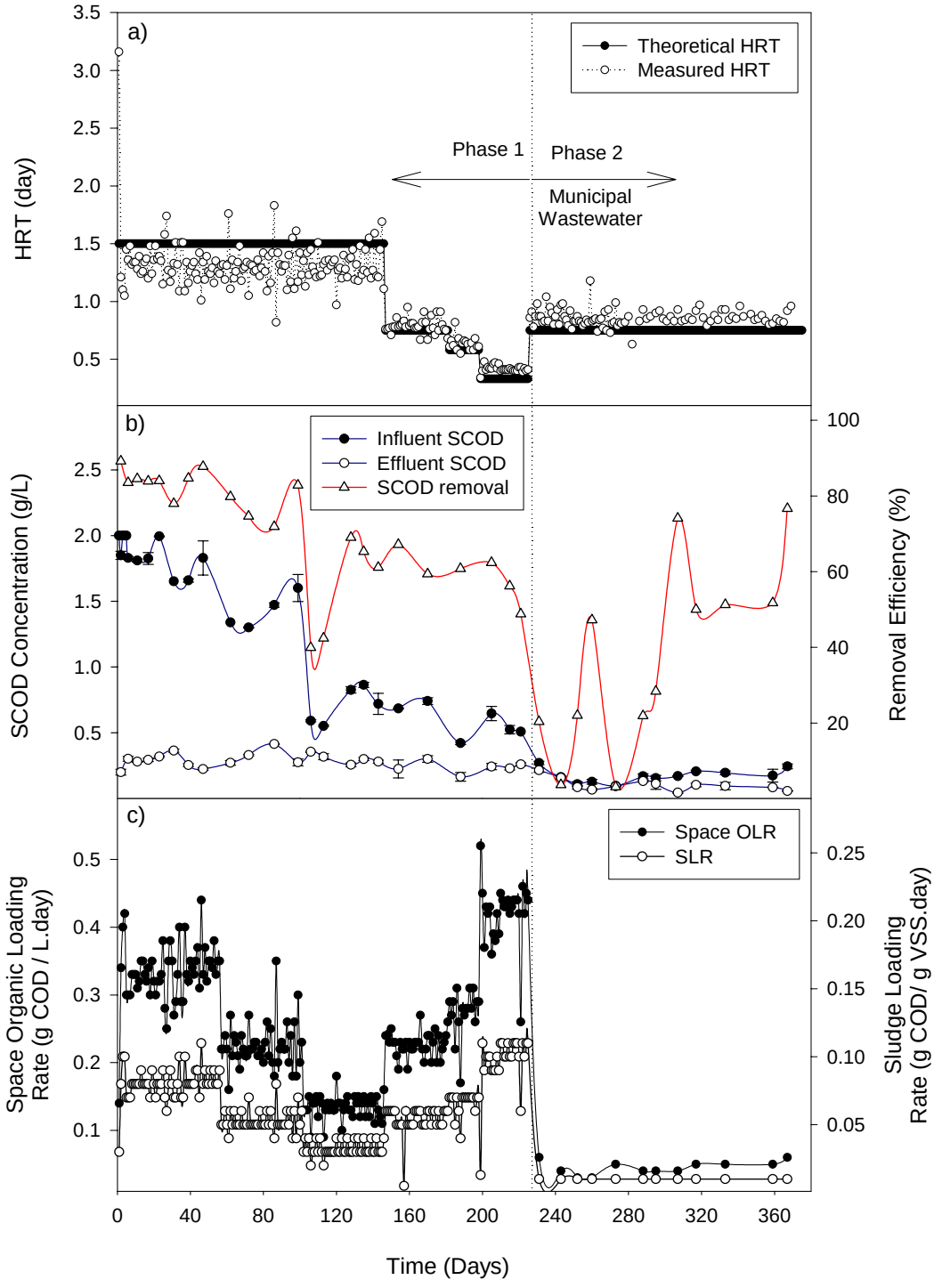


Figure 4.15. The operational conditions and the results of the experiments conducted with R1.

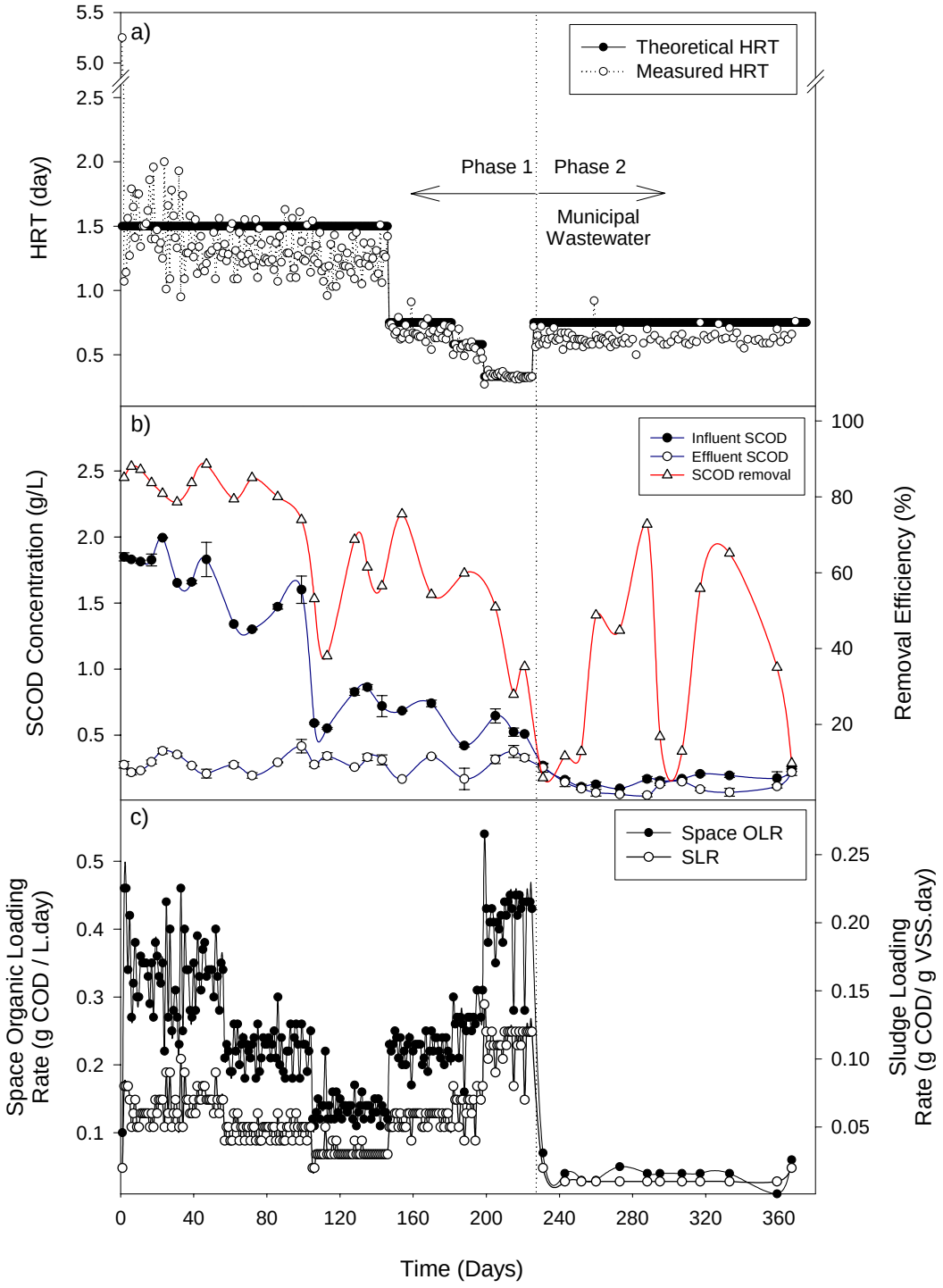


Figure 4.16. The operational conditions and the results of the experiments conducted with R2.

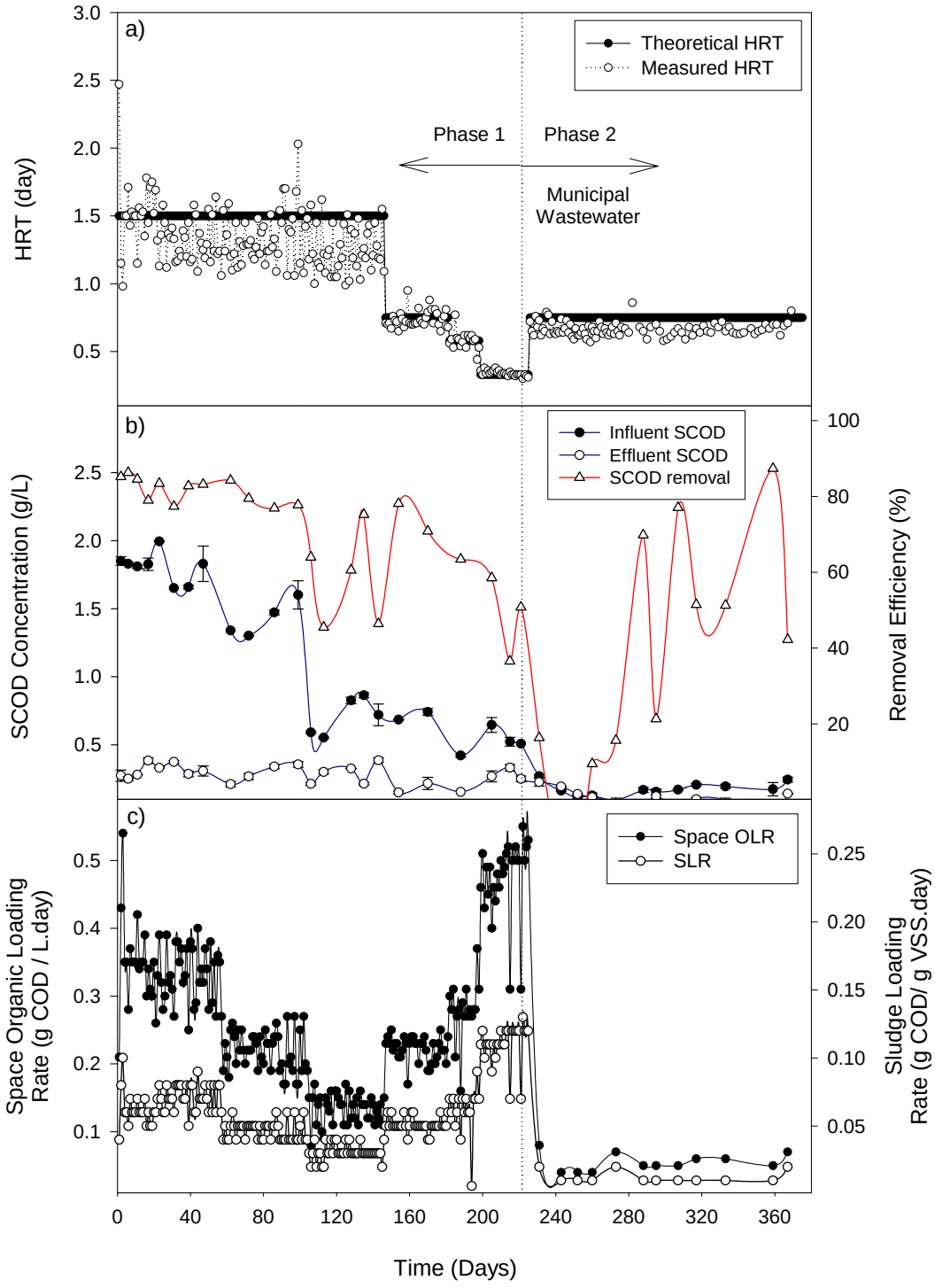


Figure 4.17. The operational conditions and the results of the experiments conducted with R3.

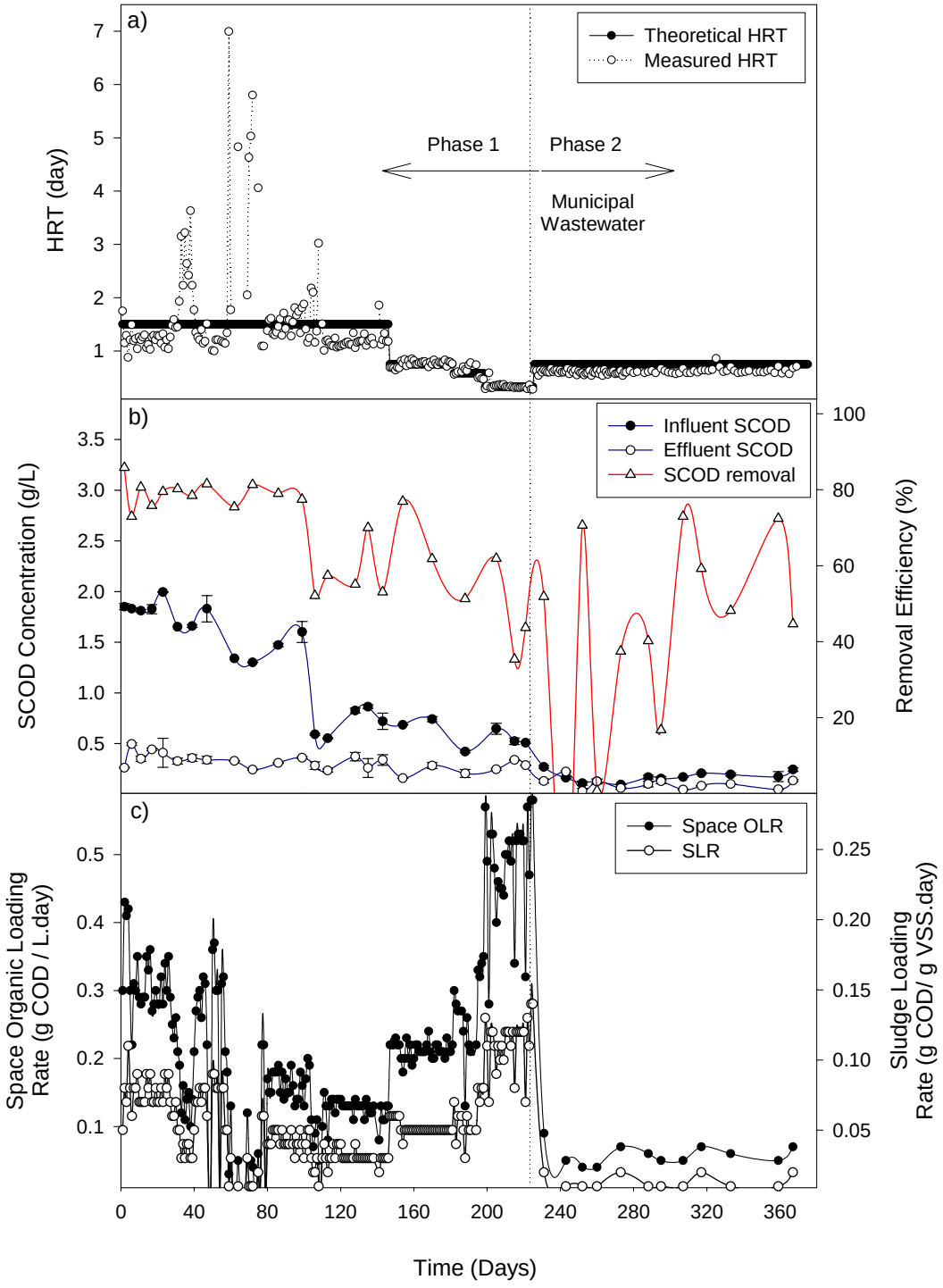


Figure 4.18. The operational conditions and the results of the experiments conducted with R4.

Table 4.9. The SCOD removal efficiencies of the USB reactors during Phase 1.

Days	Operating parameters			SCOD removal efficiency* (%)			
	HRT (days)	Influent SCOD (mg/L)	SOLR (g COD/L.day)	R1	R2	R3	R4
1-30	1.5	1800-2000	0.25-0.46	84-89	81-88	79-86	73-86
31-58	1.5	1600-1800	0.23-0.39	78-88	79-89	77-83	79-82
59-104	1.5	1300-1600	0.19-0.26	72-83	74-85	77-84	75-81
105-146	1.5	600-850	0.11-0.17	40-69	38-69	46-75	52-70
147-181	0.75	650-750	0.21-0.25	59-67	54-76	71-78	62-77
182-198	0.58	400-650	0.25-0.31	61	60	63	51
199-225	0.33	500-650	0.28-0.45	49-62	28-51	37-59	35-62

* The ranges indicate the minimum and maximum removal efficiencies achieved during the corresponding operational conditions.

As seen in Table 4.9, the influent SCOD concentration was decreased down to 1300-1600 mg/L by Day 59. Till Day 104, the performances of all reactors were more or less the same in terms of SCOD removal efficiencies, ranging within 72-89%. Achieving removal efficiencies greater than 80%, the strength of the wastewater was decreased to a range of 600-850 mg SCOD /L on Day 105. It was observed that the removal efficiencies of all reactors decreased down to 38-52% with the decrease in the strength of wastewater. The sudden decrease in the removal efficiencies of the reactors on Days 106 and 113 is also illustrated in Figures from 4.15b to 4.18b. To increase the SOLR, HRT was decreased from 1.5 to 0.75 day on Day 147. The decrease in the HRT resulted in the increase of SCOD removal efficiencies up to 67-78% between Days 147-181. Therefore, HRT values were decreased to 0.58 day and then to 0.33 day on Day 182 and Day

199, respectively. In accordance, influent SCOD was decreased down to 400-650 mg/L (Days 182-225). However, with the decrease of HRT from 0.75 day down to 0.33 day, all reactors displayed decreasing (and fluctuating) performances in terms of SCOD removal (Days 182-225, in Figures 4.15b - 4.18b). At an HRT of 0.33 day, R1, R2, R3 and R4 displayed SCOD removal efficiencies of 49-62, 28-51, 37-59 and 35-62%, respectively (Table 4.9).

The test reactors displayed significant removal efficiencies except for HRTs of 0.58 and 0.33 day (Table 4.9). This indicated that under optimum operational conditions the cultures in the test reactors performed significant activities despite of the aeration. In other words, under alternating cyclic anaerobic / aerobic / microaerobic conditions (R2 and R3) and microaerobic / aerobic conditions (R4), the cultures of test reactors operated well. They also displayed significant SOUR and SMA activities indicating their survival despite of the mentioned conditions (Section 4.2.3.2). Therefore, it can be stated that combined cultures were developed.

SCOD removal efficiency data in Table 4.9 might indicate that during Phase 1 almost similar removal efficiencies were obtained among the test and anaerobic control reactors. However, the sludge amount in the reactors should be considered while comparing the treatment performances. Therefore, the ratio of SCOD removal efficiency to VSS content was evaluated for each reactor (i.e. SCOD removal (%) / g VSS). The calculated ratios of the test reactors were later normalized with the ratio of anaerobic control reactor in order to make a better removal efficiency comparison. The results are illustrated in Figure 4.19. It is observed in Figure 4.19 that when the influent SCOD was lower than 800 mg/L, combined cultures developed in R2, R3 and R4 displayed better performances compared to anaerobic granules in R1.

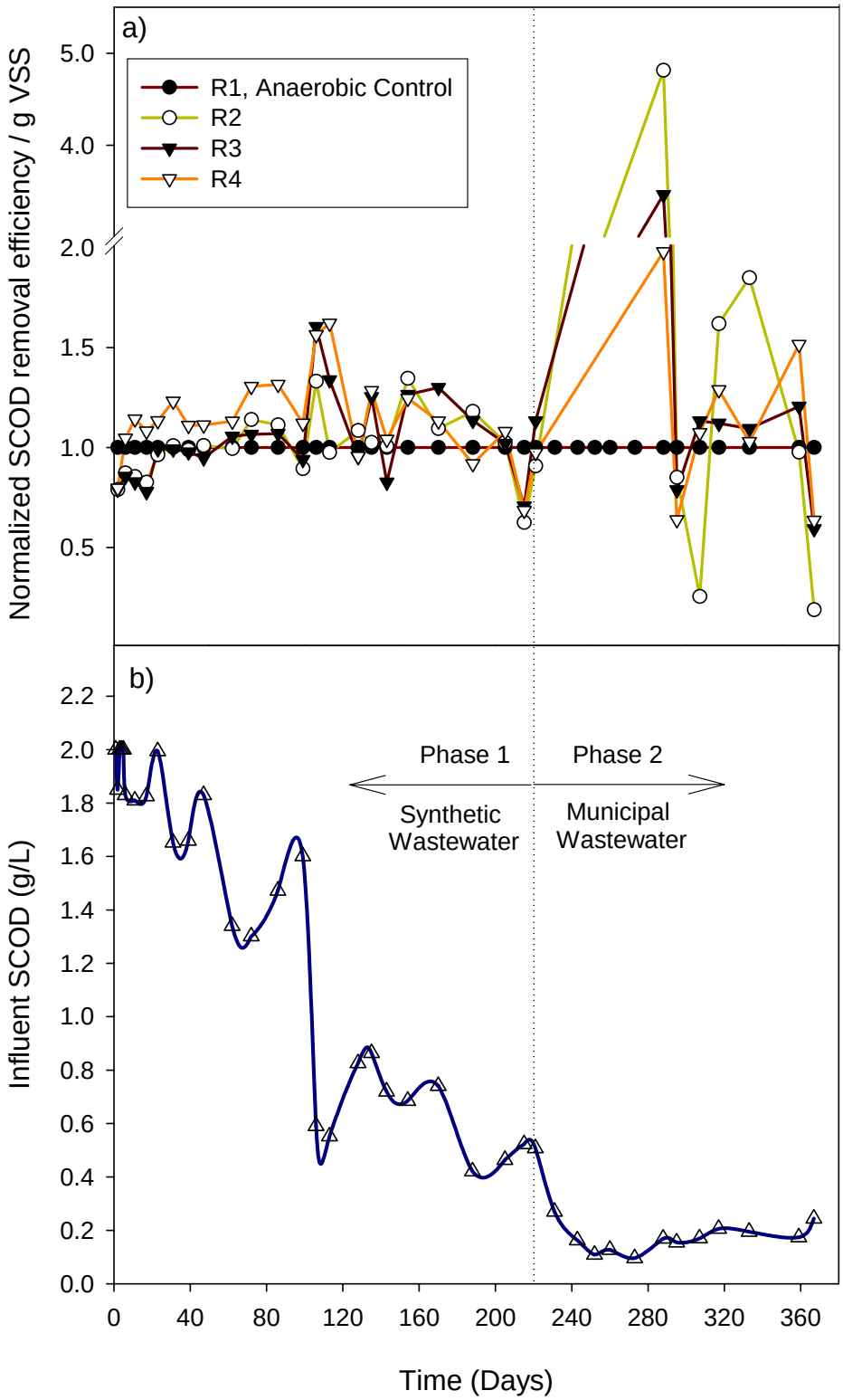


Figure 4.19. The normalized SCOD removal efficiency / g VSS values of test reactors for comparison with anaerobic control reactor.

ANOVA performed for the ratio values (SCOD removal efficiency / g VSS) also indicated that combined cultures developed in all test reactors were not statistically different in terms of SCOD removal efficiency per g VSS (Table D.2, Appendix D). Combined cultures developed under varied aeration conditions displayed similar performances. In other words, aeration periods of 4 hours/day (R2), in every other day (R3) or continuously during 24 hours (R4) did not change the removal efficiency of the combined cultures for low strength wastewaters with SCOD strength of 400-2000 mg/L. However, it is for sure that combined cultures developed under varied aeration conditions and from a mixture anaerobic granular and suspended aerobic cultures had greater removal efficiencies compared to the anaerobic granules alone especially for SCOD values lower than 800 mg/L (Figure 4.19). It can be concluded that the use of combined cultures for treatment of synthetic wastewater with influent SCOD of 400-2000 mg/L (Phase 1) under the studied conditions was advantageous compared to the anaerobic granules.

Phase 2 (Low Strength Wastewater Application -municipal wastewater-): The results of Phase 1 indicated that for the influent SCOD values of 650-750 mg/L the greatest SCOD removal efficiencies were achieved when the HRT was set as 0.75 day (Table 4.9). Thus, during Phase 2 (Days 226-370) municipal wastewater was fed to the reactors at an HRT of 0.75 day (Figures 4.15a-4.18a). The wastewater had influent SCOD concentrations ranging from 155 to 244 mg/L (Figures 4.15a-4.18a). In fact, this range resembled the weak strength municipal wastewater rather than a typical municipal wastewater of medium strength (COD of 400 mg/L, Tchobanoglous and Burton, 1991). At such low influent SCOD values, all the reactors displayed unstable SCOD removal performances. As seen in Figures 4.15b, 4.16b, 4.17b and 4.18b, the SCOD removal efficiencies were fluctuating in the range of 22-77, 10-77, 21-88 and 17-73% for R1, R2, R3 and R4, respectively. The similar fluctuating trend was also observed for the normalized values of SCOD removal efficiencies per g VSS (Figure 4.19). This fluctuation might be also due to the fluctuating and greater COD/BOD_5 ratio values of the studied municipal wastewater (1.43-2.92) than the typical value of

1.14, which indicates the low biodegradability (Tchobanoglous and Burton, 1991). However, despite of that, combined cultures (in R2, R3 and R4) displayed better SCOD removal performances compared to anaerobic cultures (R1) for most of the time (Figure 4.19).

The performances of the reactors or cultures were also assessed in terms of their BOD_5 removal efficiencies. The results of the BOD_5 analyses were given in Table 4.10. Due to the experimental errors, the results of the analyses performed during Days 226-287 were not given in Table 4.10.

Table 4.10. The influent and effluent BOD_5 concentrations and the removal performances of the USB reactors.

Days	Influent BOD_5 (mg/L)	Removal efficiencies (%)				Effluent BOD_5 values (mg/L)*			
		R1	R2	R3	R4	R1	R2	R3	R4
288	118 ± 6	58 ± 0	67 ± 2	84 ± 2	83 ± 0	50	39	19	20
295	53 ± 0	25 ± 9	35 ± 5	60 ± 13	60 ± 4	40	40	21	21
307	90 ± 4	56 ± 3	74 ± 3	84 ± 0	83 ± 3	40	23	14	15
317	94 ± 0	52 ± 4	44 ± 3	73 ± 0	77 ± 2	45	53	25	22
333	112 ± 2	42 ± 2	41 ± 3	79 ± 2	79 ± 3	65	66	23.5	23.5
359	93 ± 2	20 ± 2	59 ± 4	73 ± 0	78 ± 4	74	38	25	20.5
367	91 ± 5	30 ± 2	44 ± 4	74 ± 2	77 ± 2	64	51	24	21
Ave.	93 ± 21	40 ± 15	52 ± 15	75 ± 9	76 ± 8	54	44	21.5	20.5

* Standard deviations were in the range of 3-8 mg/L.
Ave: Average

As seen in Table 4.10, for influent BOD_5 values of 53-118 mg/L, R1, R2, R3 and R4 had average BOD_5 removal efficiencies of 40, 52, 75 and 76%, respectively. It was concluded as in Phase 1 that in treatment of municipal wastewater all the combined cultures developed under varied aeration conditions were more advantageous compared to the anaerobic granular cultures in terms of BOD_5 removal. Normalized ratio values (BOD_5 removal efficiency / g VSS) calculated for each reactor also indicated the better performances of the combined cultures developed in R2, R3 and R4 compared to anaerobic granular cultures (R1) (Figure 4.20). Table 4.10 and Figure 4.20 revealed that the combined cultures developed in R3 and R4, which were aerated in every other day or continuously, respectively, displayed almost similar BOD_5 removal performances. On the other hand, the removal efficiencies of combined cultures in R3 and R4 were greater than those of in R2 (aerated 4 hours/day). Same comment was also valid for the normalized BOD_5 removal efficiency / g VSS ratio values for most of the time (Figure 4.20). Thus, it was concluded that aeration (by 10 mL/min) during either in every other day or continuously resulted in the development of combined cultures with similar performances. To develop combined cultures with high performances under the studied conditions, aeration applied in every other day was sufficient rather than the continuous aeration.

In spite of the greater removal efficiencies (75-76%) achieved by combined cultures than those of anaerobic granules, efficiency values were comparably lower than those conventional aerobic systems, which are reported as 85-95% for a typical activated sludge system treating domestic wastewater (Tchobanoglous and Burton, 1991). However, the average effluent BOD_5 values of R2, R3 and R4 were 44, 21.5 and 20.5 mg/L, respectively, which are below the effluent discharge standards of Turkey set as 45 mg/L for 24-hr composite samples of treated domestic wastewaters (SKKY, 1988). Effluent BOD_5 values of R3 and R4 were also below the European Union effluent discharge standards set as 25 mg/L (EU, 1991).

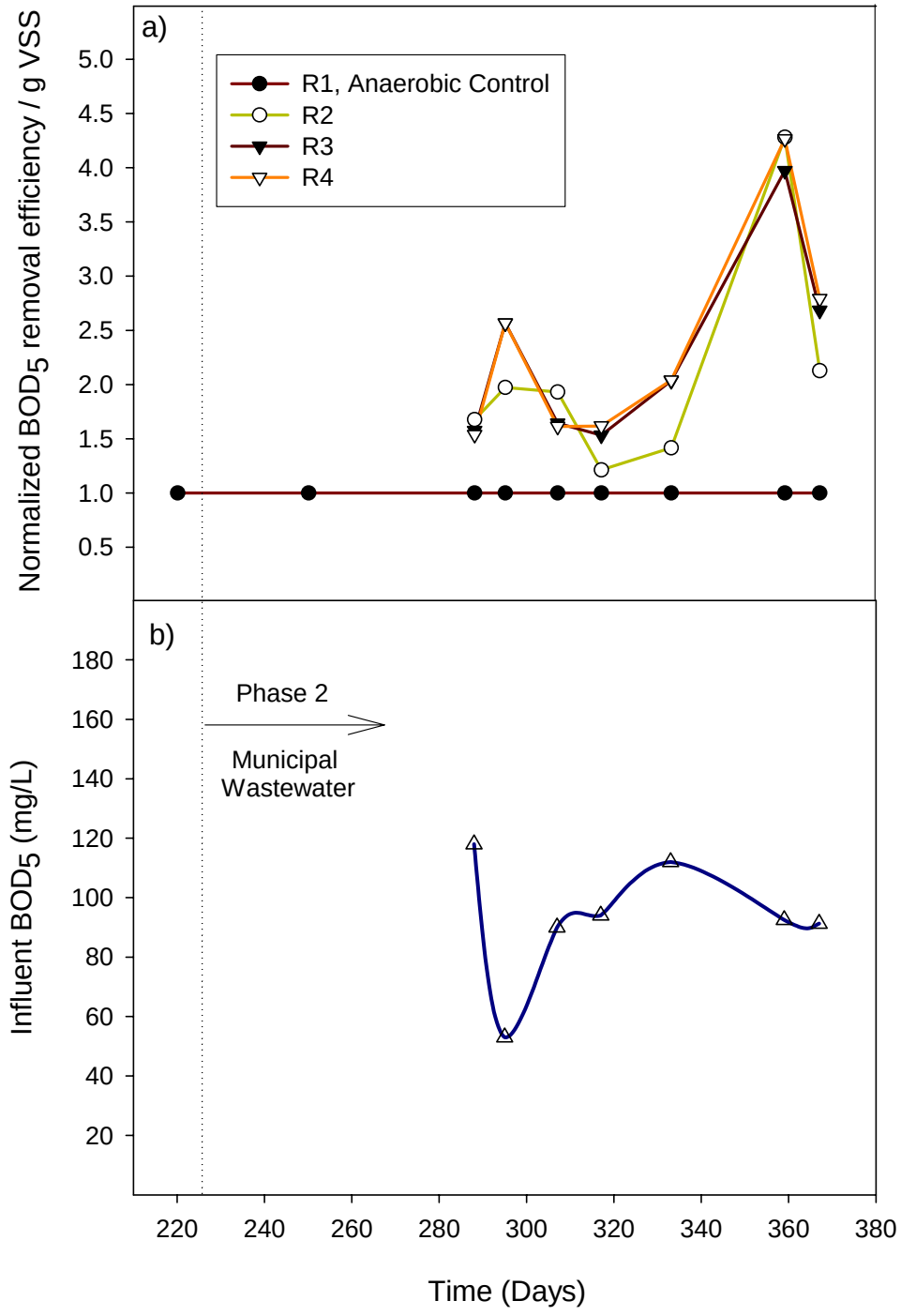


Figure 4.20. The normalized BOD_5 removal efficiency / g VSS values of test reactors for comparison with anaerobic control reactor.

Experiments revealed that combined cultures could be developed in USB reactors under aerobic / microaerobic and alternating (cyclic) anaerobic / aerobic and/or microaerobic conditions. Comparing with the anaerobic granular systems, the use of combined cultures would be an option in low strength wastewater treatment.

4.2.3.2. Physical Characteristics and Microbial Activity

Physical Characteristics: It was visually observed that the ratio of the brown to black cultures were highest in R4 which was aerated continuously, while it was the lowest in R2 (4 hours of aeration/day). The brown or yellow color was related with the aerobic cultures while black color with anaerobic cultures (Tay et al., 2004a; Hu et al., 2005a). Therefore, increasing brown to black culture ratio was attributed to the aerobic culture population escalating with the increasing aeration period. Hu et al. (2005a) observed the color change in their study where aerobic granules were developed from anaerobic granules under aerobic conditions. They defined the color change as the gradual phasing out (disintegration) of the anaerobic granules due to aerobic conditions and the further recombination of the aerobic suspended solids. However, in this study, based on the significant SMAs of the developed cultures through the experimental period and physical characteristics comparable to granules (mentioned below), it can be said that anaerobic granules were not phased out. The possible color difference between test reactors might be due to the amount of aerobic cultures. The aeration period might have affected the amount of the aerobic cultures cultivated in the reactors, which explains the highest amount of brown colored-cultures in R4. It was observed that floc-like brown colored-cultures (i.e. aerobes) were located around the granules but not distributed separately in the supernatant. The brown-colored flocs around the surface of the granular cultures were clearly observed in R4 and also in R3. The adhesion of the aerobic cultures and attachment on granules might be regarded as an effective strategy of cells against environmental stresses such as

alternating DO conditions and starvation (Bossier and Verstrate, 1996; Tay et al., 2004a), the latter accomplished during municipal wastewater application.

The SVI and settling velocities of the combined cultures measured at the end of 370 days are given in Table 4.11. As seen in Table 4.11, the cultures in R2, R3 and R4 had average settling velocities of 27.4, 26.3 and 25.1 m/hr, which are within the range of the values given for anaerobic granules (20-50 m/hr, Schmidt and Ahring, 1996) and aerobic granules (22-77 m/hr, Liu and Tay, 2004; Hu et al., 2005a) (Table 4.6). SVI values, namely, 54.4, 63.3 and 66.4 mL/g for R2, R3 and R4 are greater than the SVI values of anaerobic granules (35-45 mL/g, Noyola and Moreno, 1994) but well comparable to those of aerobic granules (50-85 mL/g, Tay et al., 2001a) (Table 4.6).

Table 4.11. The physical characteristics of the combined cultures.

Reactor type	Aeration period (10 mL/min)	Settling velocity (m/hr)		SVI (mL/g)
		Range	Average	
R1	-	26-45	34.4 ± 6	36
R2	4 hours/day	18-50	27.4 ± 10	54.4
R3	Every other day	16-50	26.3 ± 7	63.3
R4	Continuously	14-60	25.1 ± 11	66.4

Table 4.11 also indicates that as the aeration period increased, the settleability of the combined cultures slightly decreased. The seeding of the test reactors with both granular anaerobic and suspended aerobic cultures resulted in the increased SVI values of the anaerobic granules (from 36 up to 66 mL/g), which might be explained by the attachment of the aerobic cultures (like flocs) on the granules and in turn the decrease in the average settling velocities (from 34 to 25 m/hr).

The amount of aerobic cultures around the granules was related with the aeration periods or the conditions prevailing in the reactors. Thus, the increasing aeration periods (i.e. the increasing amount of aerobic cultures) resulted in the decreasing settling performances (Table 4.11, in the order of R2, R3 and R4). The other possible reason of decreasing SVIs might be the mechanical disintegration of the granular cultures into small sized-granules, which is probably enhanced by the aeration period. The formation of small granules of varied sizes might explain the increasing settling velocity ranges and increasing standard deviations proportional to the increased aeration periods (Table 4.11). The similar increasing SVI values with the increasing aeration period was also observed in a study where the anaerobic granules were exposed to varied DO levels (from 0.5 to 8 mg/L) (Shen and Guiot, 1996). However, they explained the impairment of the granule settleability with the float off the sludge bed and in turn its washout, which was not the case in this study.

Microbial Activity: During operation period of 370 days SMA and SOUR analyses were performed, to investigate the activities of the combined cultures and in turn their survival under alternating conditions. The results of the SOUR and SMA analyses are given in Tables 4.12 and 4.13, respectively. Because the reactors were operated at varying influent SCOD, SOLR and HRT values through the operation period, SMA and SOUR values were evaluated among the reactors independent of time. Therefore, comparison among the reactors was performed considering the ranges of the SMA and SOUR activities but not with respect to their trends.

As seen in Table 4.12, the combined cultures developed in R2, R3 and R4 had approximate SOUR activities of 7-28, 6-23 and 6-20 mg DO/g VSS.hr, respectively. These values were comparable and slightly greater than that of activated sludge given as 10-19.2 mg DO/g VSS.hr (Lens et al., 1995; Zitomer and Shrout, 2000). This indicated that combined cultures in all test reactors were constituted of aerobic cultures. Besides, the SOUR values resembling the

activated sludge rather than the aerobic granules (29-90 mg DO/g VSS.hr, Tay et al., 2004a) indicated that the aerobic cultures were not in the form of granules but most probably in the form of flocs (surrounding the granular anaerobes due to brown colored-granules observed).

Table 4.12. The results of the SOUR analyses of USB reactors.

Phases	Days	SOUR (mg DO/g VSS.hr)*			Influent SCOD (mg/L)	HRT** (days)
		R2	R3	R4		
Phase 1 (Synthetic WW)	46	6.83	5.50	5.90	1800	1.5 (36)
	98	7.85	11.63	13.80	1600	1.5 (36)
	155	7.47	13.55	19.39	690	0.75 (18)
	219	21.53	21.77	17.90	500	0.33 (8)
Phase 2 (Municipal WW)	265	28.09	22.83	19.81	130	0.75 (18)
	318	26.28	14.59	8.67	200	0.75 (18)
	370	9.62	6.131	6.94	250	0.75 (18)

* Standard deviations are in the range of 0.01-0.13.
** Values given in parentheses are in terms of hour.
WW: wastewater

In spite of the short aeration period of 4 hours, combined cultures developed in R2 displayed high and even greater range of SOUR values (7-28 mg DO/g VSS.hr) than those of in R3 and R4. This might be explained by the increased OUR activities of the cultures under environmental stress conditions such as prolonged anaerobic and/or microaerobic conditions (Yerushalmi et al., 2002). The average OUR values of the cultures in R2, R3 and R4 were 0.2406, 0.1623 and 0.1646 mg DO/L.min, respectively. The similar increased OUR activity was also observed

for 60% oxygen-fed combined granules in semi-continuous reactor study 4 (Section 4.1.4.3).

Table 4.13. The results of the SMA analyses of USB reactors.

Phases	Days	SMA (mL CH_4/g VSS.hr)				Influent SCOD (mg/L)	HRT* (days)
		R1	R2	R3	R4		
Phase 1 (Synthetic WW)	46	40.00	24.32	17.08	10.61	1800	1.5 (36)
	98	17.01	18.56	29.02	34.52	1600	1.5 (36)
	155	20.88	25.99	13.72	26.80	690	0.75 (18)
	219	29.55	49.83	24.48	15.73	500	0.33 (8)
Phase 2 (Municipal WW)	265	20.69	61.57	32.05	33.67	130	0.75 (18)
	327	17.94	31.02	18.12	25.54	200	0.75 (18)
	370	8.62	18.18	24.20	23.15	250	0.75 (18)

* Values given in parentheses are in terms of hour.
WW: wastewater.

As seen in Table 4.13, SMA values of the cultures in R1, R2, R3 and R4 were approximately in the range of 9-40, 18-62, 14-32, 11-35 mL CH_4/g VSS.hr, respectively. The activities of the combined cultures in R3 and R4 were comparable to those of anaerobic granules in R1 and to the typical SMA of anaerobic granules reported as 10.5-44 mL CH_4/g VSS.hr (Schmidt and Ahring, 1996). However, SMA values of cultures in R2 were greater than those of anaerobic granules in R1 (Table 4.13) and than the reported values occasionally. Limited aeration (4 hours/day) might have provided a certain level of mixing and increased the mass transfer between the liquid (thus substrate) and granules

(without disturbing the granular structure). This might have resulted in the increased layer of active anaerobic culture in the granules and thus greater SMA values than those of non-aerated control granules (R1). Nevertheless, SMA results revealed that in spite of the microaerobic and aerobic conditions achieved during aeration periods, all the combined cultures produced significant amount of methane. The methanogens might have been located deep inside the granules, where the oxygen could not diffuse. Thus, after the oxygen diffusive layer of 200-400 μm (Kurosawa and Tanaka, 1990; Lens et al., 1995), anaerobic cultures were supported inside to operate.

SMA and SOUR analyses indicated that in spite of the alternating cyclic anaerobic / aerobic and/or microaerobic conditions achieved in R2 and R3, anaerobic and aerobic cultures survived and even operated well under the studied conditions. Similarly, the seed cultures in continuously aerated R4 survived under the microaerobic/aerobic conditions and displayed similar microbial characteristics as the anaerobic and aerobic cultures. Therefore, it can be stated that combined cultures which survived and operated under anaerobic / aerobic and/or microaerobic and microaerobic/aerobic conditions were developed in all of the test reactors.

4.2.4. Comparison of the Aeration Requirement of the USB Reactors with the Activated Sludge Process

In this section, the objective was to investigate whether the USB reactors with combined cultures could be an alternative to the widely used biological system, i.e. conventional activated sludge systems, for municipal wastewater treatment. Therefore, experimental results of the USB reactor studies were considered in terms of air required by the combined cultures for municipal wastewater treatment. In other words, this section covered the aeration requirement of

combined cultures developed in USB reactor studies 2 and 3 (Sections 4.2.2 and 4.2.3).

As mentioned in USB reactor study 2 (Section 4.2.2), the USB reactor (seeded with suspended anaerobic and aerobic cultures and aerated 4 hours/day) had BOD_5 removal efficiency of 66-68% leading to the effluent value of 31-36 mg/L. The test reactors R2, R3 and R4 operated in USB reactor study 3 (Section 4.2.3) achieved average BOD_5 removal efficiencies of 52, 75 and 76% with effluent values of 44, 21.5 and 20.5 mg/L, respectively. The effluent BOD_5 concentrations of all USB test reactors were below the discharge standards (45 mg/L, SKKY, 1988) which were also comparable to those of conventional activated sludge systems. However, USB reactors, in other words combined cultures, should be also considered in terms of the following parameters such as volume of air applied per mass of BOD_5 applied to the reactor (m^3/kg), per BOD_5 removed (m^3/kg), and per volume of wastewater treated (m^3/m^3). It should be noted that to be able to make a comparison among the reactors they should have the same units in terms of air applied per day. The USB reactor in study 2 and USB reactors R2 and R4 in study 3 were aerated on daily bases, while R3 was aerated every other day (during one day in two days-time). The aeration period of R3 was, therefore, considered as 12 hours/day, which means 24 hours per 2 days. The results and the comparison of the USB reactors with conventional activated sludge systems in terms of volume of air applied per BOD_5 removed and applied and per volume of municipal wastewater treated are given in Table 4.14. An example calculation is given in Appendix F.

It is stated that for each kg of BOD_5 removed 50-95 m^3 air should be applied to the conventional activated sludge systems with diffused aeration (Corbitt, 1990). As seen in Table 4.14, the ratio of air volume per removed BOD_5 values of all test reactors except the one operated in study 2 were too much higher than the values reported in the literature. However, it should be noted that the air flow rate of 10 mL/min was the lowest flow rate that could be applied during the experiments

without clogging of the diffusers and could be measured by the flow meter. Therefore, this is not necessarily the minimum required flow rate to be applied to the USB reactors for combined culture development and municipal wastewater treatment. Lower aeration rates could also be sufficient.

Table 4.14. The comparison of the reactors with conventional activated sludge systems in terms of air volume applied per kg BOD_5 applied and removed and per volume of municipal wastewater treated.

Reactors		Air / BOD_5 applied (m^3/kg)		Air / BOD_5 removed (m^3/kg)		Air / WW (m^3/m^3)
		Range	Average	Range	Average	Range
USBR study 3	R2	122-147	136	172-357	279	13-15
	R3	249-349	304	333-464	400	29-32
	R4	468-692	602	569-883	768	44-50
USBR study 2		24-70	45	61-106	80	3-6
Conventional activated sludge systems*	1	92		-		-
	2	-		50-95		-
	3	-		-		3.75-15

* 1) Benefield and Randall, 1985; 2) Corbitt, 1990; 3) Tchobanoglous and Burton, 1991.
USBR: Upflow sludge blanket reactor, WW: wastewater treated.

The volume of air per removed (or applied) BOD_5 values of conventional activated sludge systems are determined considering the optimum oxygen amount required by the microorganisms. DO concentrations in activated sludge system range within 1.5-4.0 mg/L with an optimum of 2.0 mg/L (Tchobanoglous and Burton, 1991). The higher oxygen doses or amounts result in excess DO and

higher operational costs therefore not desired. However, in this study the aeration rate was not determined considering the required oxygen amount. Instead, the air flow rate, which was the lowest rate that could be applied with the air pump used, was determined based on the performance of the air pump. Therefore, excess aeration resulted in high DO levels (especially in R4 up to 5.1 mg/L) (Table 4.7).

The excess aeration can also be understood from the values of air volume applied per volume of municipal wastewater treated (Table 4.14). The USB reactor in study 2 had a greater volume compared to the ones in study 3. At equal HRT values of 0.75 day, greater volume of wastewater was therefore fed to the USB reactor, almost 2.5-3.7-fold greater than those of R2. This resulted in comparable ratios of air volume / volume of wastewater for the USB reactor in study 2 (3-6 m^3/m^3) as those of conventional activated sludge systems (3.75-15 m^3/m^3).

The air volumes required in test reactors were also considered for synthetic wastewater application to indicate the excess aeration (Table 4.15). As seen in Table 4.15, the ratio values of air volume per kg SCOD applied (or removed) and per wastewater volume significantly decreased during synthetic wastewater application. Such remarkable differences in ratio values calculated for municipal and synthetic wastewater applications were attributed to the influent BOD_5 and/or SCOD values (Tables 4.14 and 4.15). The influent SCOD concentration of the synthetic wastewater applied to the USB reactors in studies 2 and 3 were in the range of 570-3500 and 400-2000 mg/L, respectively. Lower influent SCOD and BOD_5 concentrations of municipal wastewater (155-244 mg/L and 53-118 mg/L, respectively) than those of synthetic wastewater indicated that lower aeration rates should have been applied during municipal wastewater application. However, as already mentioned the studied aeration rate (10 mL/min) was the lowest rate that could be applied with the air pump and diffuser system used.

In addition to the excess aeration, high ratios of air volume / removed BOD_5 (or applied) might be due to the limitation in oxygen mass transfer. The USB reactors

were aerated with a porous air stone located at the bottom of the reactor. Resembling the diffused aeration, the air / removed BOD_5 values of reactors were compared with those of the conventional activated sludge systems with diffused aeration. It is believed that the air applied to the reactors was not thoroughly transferred to the reactor content. The resultant DO concentrations in the reactor contents of USB reactors were most probably achieved by the portion of the air (oxygen) that could partially be transferred. With a well operating diffuser system, greater mass transfer could have been achieved at lower aeration rates.

Table 4.15. The air volume applied per kg SCOD applied and removed and per volume of synthetic wastewater treated.

Reactors		Air / SCOD applied (m^3/kg)		Air / SCOD removed (m^3/kg)		Air / WW (m^3/m^3)
		Range	Average	Range	Average	Average
USBR study 3	R2	7-29	14	8-64	24	5
	R3	21-111	46	25-191	71	15
	R4	43-217	119	50-379	182	38
USBR study 2		4-12	7	6-13	8	2

USBR: Upflow sludge blanket reactor, WW: wastewater treated.

CHAPTER 5

CONCLUSIONS

Based on the results of this study, the following conclusions could be made.

- Combined cultures which were composed of both anaerobic and aerobic microorganisms could be developed and operated in semi-continuous and USB reactors under alternating conditions. Combined granules could also be developed from a mixture of suspended anaerobic and aerobic cultures.

- Combined granules developed in the semi-continuous reactors under alternating cyclic anaerobic / aerobic and microaerobic conditions and aerobic / microaerobic conditions had median sizes of 1.28 and 1.86 mm and average settling velocities of 31.0 ± 7.4 and 39.2 ± 8.3 m/hr, respectively. They had noteworthy SOUR (6-47 mg DO/g VSS.hr) and SMA (14-42 mL CH_4/g VSS.hr) values, which were comparable to those of aerobic and anaerobic granules, respectively. Similarly, combined granules developed in the USB reactor (aerated at 10 mL/min for 4 hours/day) under alternating anaerobic / microaerobic conditions resembled the granular cultures in terms of their sizes (0.4-1.92 mm) and settling velocities (12-60 m/hr). The significant SMA (11-77 mL CH_4/g VSS.hr) and SOUR (10-75 mg DO/g VSS.hr) values of the combined granules/cultures indicated their constituents, i.e. both anaerobic and aerobic cultures.

- In spite of the alternating cyclic conditions, the survival and the activity levels of the anaerobic and aerobic cultures constituting the combined

granules supported the hypothesized granulation mechanism: location of the cultures based on their physiological characteristics. Aerobic cultures were located at the more oxidized outer parts, while the anaerobes in the oxygen-free inner parts of the combined granules (i.e. shielding effect) as survival mechanism.

- Combined granulation of suspended anaerobic and aerobic cultures was highly dependent on the interaction between the anaerobic and aerobic cultures, the oxygen dose applied, the peculiarity of the cyclic operation and in turn the resultant alternating cyclic conditions. Alternating cyclic anaerobic / aerobic and microaerobic conditions or aerobic / microaerobic conditions of adequate periods affected the aerobic cultures which triggered the granulation and shortened the granulation period (2-3 weeks in semi-continuous reactors). The slowly proceeding granulation (4-5 months) and the existence of floc-like pellets in the USB reactor were attributed to the insufficient aeration (strength and period), low DO conditions and in turn the alternating cyclic anaerobic / microaerobic conditions. It might be the anaerobic cultures that mostly determined the formation of compact granules in USB reactor.

- Combined granules developed in semi-continuous reactors exhibited similar removal performances as those of anaerobic and aerobic granules. However, they were still superior because they also overcame the drawbacks of both anaerobic and aerobic granules such as the need for long start-up and low stability, respectively. Between the combined granules fed with oxygen doses of 60 and 120% of the total COD added, the 60% oxygen-fed combined granules (developed under alternating anaerobic / aerobic and microaerobic conditions) might be advantageous due to their similar microbial activities as those of 120% oxygen-fed combined granules but less oxygen requirement.

- Combined cultures were also developed from anaerobic granular and suspended aerobic cultures in USB reactors. SMA (11-62 mL CH_4/g VSS.hr) and SOUR (6-28 mg DO/g VSS.hr) values of the combined cultures were comparable to those of anaerobic granules and activated sludge, respectively. Their average settling velocities (25.1-27.4 m/hr) and SVI values (54.4-66.4 mL/g) were well within the range of the values given for granular cultures. Both microbial and physical characteristics of the combined cultures indicated the conservation of the granular structure of the anaerobic granules and most probably their entrapment by aerobic cultures.

- The use of combined cultures was found to be advantageous compared to the anaerobic granules for the treatment of low strength wastewaters. The aeration period did not affect the performances of the cultures during synthetic wastewater application (influent SCOD of 400-2000 mg/L). During municipal wastewater treatment, combined cultures exhibited average BOD_5 removal efficiencies of 52-76%, which were greater than those of anaerobic granules (40%). Among the cultures developed, combined cultures which were aerated every other day were selected as the most advantageous due to their higher removal efficiencies, slightly better settling characteristics and lower oxygen requirement. Combined cultures/granules developed in USB reactor from suspended anaerobic and aerobic cultures also displayed significant BOD_5 removal efficiencies (66-68%) for the treatment of municipal wastewater at an HRT of 0.75 days.

- The use of combined cultures or granules developed in USB reactors might be proposed as an alternative to the conventional activated sludge systems for municipal wastewater treatment. They achieved the required effluent BOD_5 discharge standards and the similar effluent quality as those of activated sludge systems. The problems experienced in the activated sludge systems such as sludge bulking was not the case for combined

cultures/granules. The low effluent MLSS/MLVSS concentrations (< 20 mg/L) also overcame the high amount of sludge production and related handling problems encountered in the activated sludge systems. In addition, they had the advantages of advanced anaerobic treatment systems such as uninhibited activity and high settling characteristics as well as possible methanogenic activity comparable to those of anaerobic granules.

- The only disadvantage of combined cultures appeared to be the volume of air applied per kg of BOD_5 removed (or applied). The air volume required by combined granules/cultures developed from suspended anaerobic and aerobic cultures for each kg of BOD_5 removed (or applied) was similar to those of conventional activated sludge systems. However, it was too much higher for the combined cultures developed from anaerobic granular and suspended aerobic cultures. In spite of the comparable removal performances and SOUR activities as those of conventional activated sludge systems, high values of air requirement was attributed to the limited oxygen transfer and excess aeration. Lower aeration rates (less than 10 mL/min) could even be sufficient.

CHAPTER 6

FUTURE WORK

Regarding the results and the conclusion of this study, the following points can be recommended for future work.

- In order to investigate the spatial distribution of aerobic and anaerobic cultures in combined granules/cultures, fluorescent in situ hybridization (FISH) tests might be applied. Utilization of microelectrodes will be beneficial in determining the activity through the granules. The specific culture types constituting the granules should be identified. The effect of alternating cyclic anaerobic / aerobic conditions in terms of cycle number and intensity on the proliferation of specific cultures might be examined.

- The gas composition of the reactors should be analyzed through alternating cyclic conditions, which will indicate the activities of the cultures and the nature of the degradation mechanisms of combined cultures.

- The effect of substrate concentration on the granulation of anaerobic and aerobic cultures and formation of alternating conditions in semi-continuous reactor experiments might be examined. The effect of temperature on development of combined granules and low strength wastewater treatment might be also investigated.

- The low strength wastewater treatment and development of combined granules/cultures in USB reactors might be carried out under lower

aeration flow rates with efficiently operating diffusers and gas-solid separators. Combined granulation study should be investigated in USB reactors under cyclic alternating anaerobic / aerobic conditions.

- The use of combined granules/cultures can be better understood for treatment of many problematic recalcitrant pollutants requiring sequential treatment. The complete mineralization of highly chlorinated compounds (such as perchloroethylene, chlorobiphenlys, chlorophenols 2,3,6-trichlorobenzoic acid, 4-chloro-2-nitrophenol requiring sequential anaerobic and aerobic or anoxic treatment), or treatment of wastewaters with high sulfate and COD content; with high nutrient content (complete nitrification / denitrification, or phosphorus removal) should be studied with combined cultures/granules. The effect of using combined cultures/granules on the degradation rates might be another approach.

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APPENDIX A

CHARACTERISTICS OF THE MUNICIPAL WASTEWATER

The municipal wastewater was obtained from the effluents of the primary settling tanks of the Greater Municipality of Ankara Central Wastewater Treatment Plant. The characteristics of the wastewater is shown in Table A.1.

Table A.1. The characteristics of the municipal wastewater.

Parameter	Range	Average	Number of measurement
SCOD (mg/L)	155 - 244	187.5 ± 30	7
BOD_5 (mg/L)	53 - 118	93 ± 21	7
pH	7.27 – 7.8	7.52 ± 0.23	10
Alkalinity (mg/L)	300 - 410	360 ± 47	10
NH_4-N (mg/L)	12 – 22	16.7 ± 3	3
PO_4-P (mg/L)	4 - 10.8	6.2 ± 1	3
SS (mg/L)	17 – 76	45 ± 27	10
VSS (mg/L)	17 – 62	36 ± 18	10

APPENDIX B

THE PROPERTIES OF PRIMARY SUSBTRATES USED IN THE EXPERIMENTS, THEIR THEORETICAL COD CALCULATIONS AND COD CALIBRATION CURVE

The main properties of the primary substrates used in the experiments, namely, ethanol, glucose and HAc are given in Table B.1.

Table B.1. The main properties of the primary substrates used in the experiments.

Properties	Ethanol	HAc	D-glucose monohydrate
Chemical Formula	C_2H_5OH	CH_3COOH	$C_6H_{12}O_6.H_2O$
MW (g/mole)	46	60.05	198.17
Density (kg/L)	0.788	1.050	-
Purchased from	Sigma-Aldrich, Germany	Merck, Germany	Merck, Germany

Theoretical COD calculations were used to determine the ethanol, glucose or HAc amount to be fed to the reactors and to achieve required COD concentrations in the reactors. The theoretical COD concentrations were calculated by using the stoichiometric mineralization equations of the chemicals (ethanol, glucose and HAc) to CO_2 and H_2O (Speece, 1996).

$$C_2H_5OH + 3O_2 \rightarrow 2CO_2 + 3H_2O$$

$$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O$$

$$C_6H_{12}O_6.H_2O + 6O_2 \rightarrow 6CO_2 + 7H_2O$$

MW of oxygen: 32 g/mole

$$\frac{96\ g\ O_2}{46\ g\ C_2H_5OH} = 2.087 \frac{g\ O_2}{g\ C_2H_5OH}$$

1 g ethanol = 2.087 g COD

1 mL ethanol = 1.6446 g COD

$$\frac{192\ g\ O_2}{198.17\ g\ C_6H_{12}O_6.H_2O} = 0.969 \frac{g\ O_2}{g\ C_6H_{12}O_6.H_2O}$$

1 g glucose = 0.969 g COD

$$\frac{64\ g\ O_2}{60.05\ g\ CH_3COOH} = 1.066 \frac{g\ O_2}{g\ CH_3COOH}$$

1 g HAc = 1.066 g COD

1 mL HAc = 1.1193 g COD

In order to check the accuracy of the hand-made COD solutions, a standard substance, i.e. potassium acid phthalate (KHP), prepared with respect to the Hach Water Analyses Handbook (1998) was used. The results given in Figure B.1 indicate the high recovery of the hand-made COD solutions and in turn their applicability in the analyses. R^2 of more than 0.99 reveals the dependability of the hand-made solution.

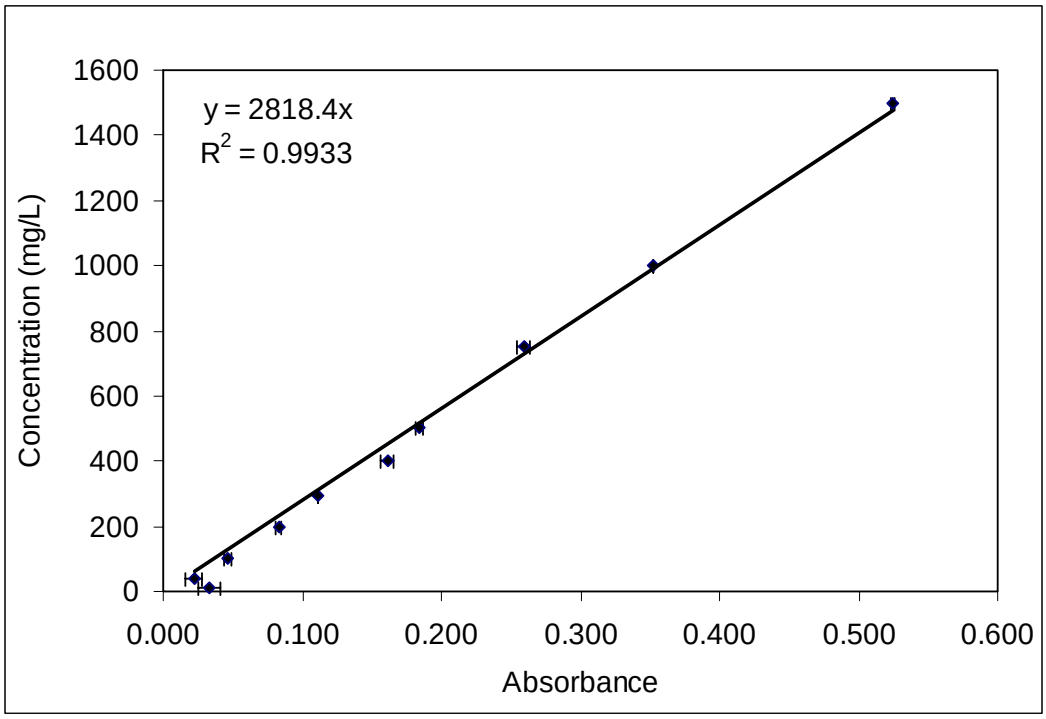

Figure B.1. The calibration curve of the hand-made COD solution.

APPENDIX C

CALCULATION OF THE OXYGEN VOLUMES APPLIED TO THE SEMI-CONTINUOUS REACTORS

Oxygen doses, which were applied in the semi-continuous reactor experiments during the continual two-day schedule, were calculated with respect to the total COD added as follows;

Example calculation for the oxygen dose of 60% of the total COD added

The total COD added in the continual two-day schedule was 50 mg.

60 % of COD = 50 mg COD × 0.60 = 30 mg O_2 = 30 × 10^{-3} g
Molecular weight (MW) of O_2 = 32 g/mol
Mol of O_2 added = 30 × 10^{-3} g / 32 g/mol = 0.9375 mmol = 0. 9375 × 10^{-3} mol
1 mol of gas = 24.05 L (at 20^0C, 1 atm)
The volume of oxygen = 0. 9375 × 10^{-3} mol × 24.05 L/mol = 22.54 mL ≈ 23 mL

Because the experiments were conducted at ambient temperature (i.e. 20^0C), in other words, oxygen additions were performed at 20^0C, the volume of the oxygen to be fed to the reactors was calculated based on that temperature.

The oxygen doses studied in the semi-continuous reactor experiments were 10, 30, 60, 100 and 120% of the total COD added (i.e. 50 mg). Following the example calculation given above, the required oxygen volumes to achieve 10, 30, 100 and 120% of the total COD added at 20^0C were calculated approximately as 4, 12, 38 and 45 mL, respectively.

APPENDIX D

THE RESULTS OF ONE-WAY ANOVA TESTS

Table D.1. One-way ANOVA results performed for the particle size data of the granules developed in semi-continuous reactor study 4.

Reactors	One-way ANOVA ($F_{0.95}$ (dF;dN))*	$F_{critical}$
60 and 120% oxygen-fed test	$F_{0.95}$ (1; 556) = 24.6661	3.85824
120% oxygen-fed test and aerobic C.	$F_{0.95}$ (1; 556) = 268.081	3.85824

* $F_{0.95}$: 95% confidence interval, dF: degree of freedom; dN: sample size; F < Fcritical: same mean, nonsignificant, C.: Control.

Table D.2. One-way ANOVA results performed for the SCOD removal efficiency per g VSS ratio values of USB test reactors in USB reactor study 3.

Reactors	One-way ANOVA ($F_{0.95}$ (dF;dN))*	$F_{critical}$
R2-R3-R4	$F_{0.95}$ (2; 66) = 2.40026	3.13592
R2-R3	$F_{0.95}$ (1; 44) = 0.09389	4.0617
R3-R4	$F_{0.95}$ (1; 44) = 2.98769	4.0617
R4-R2	$F_{0.95}$ (1; 44) = 3.46476	4.0617

* $F_{0.95}$: 95% confidence interval, dF: degree of freedom; dN: sample size; F<Fcritical: same mean, nonsignificant.

APPENDIX E

THE OPERATIONAL RESULTS OF THE TWO USB REACTORS OPERATED IN STUDY 1

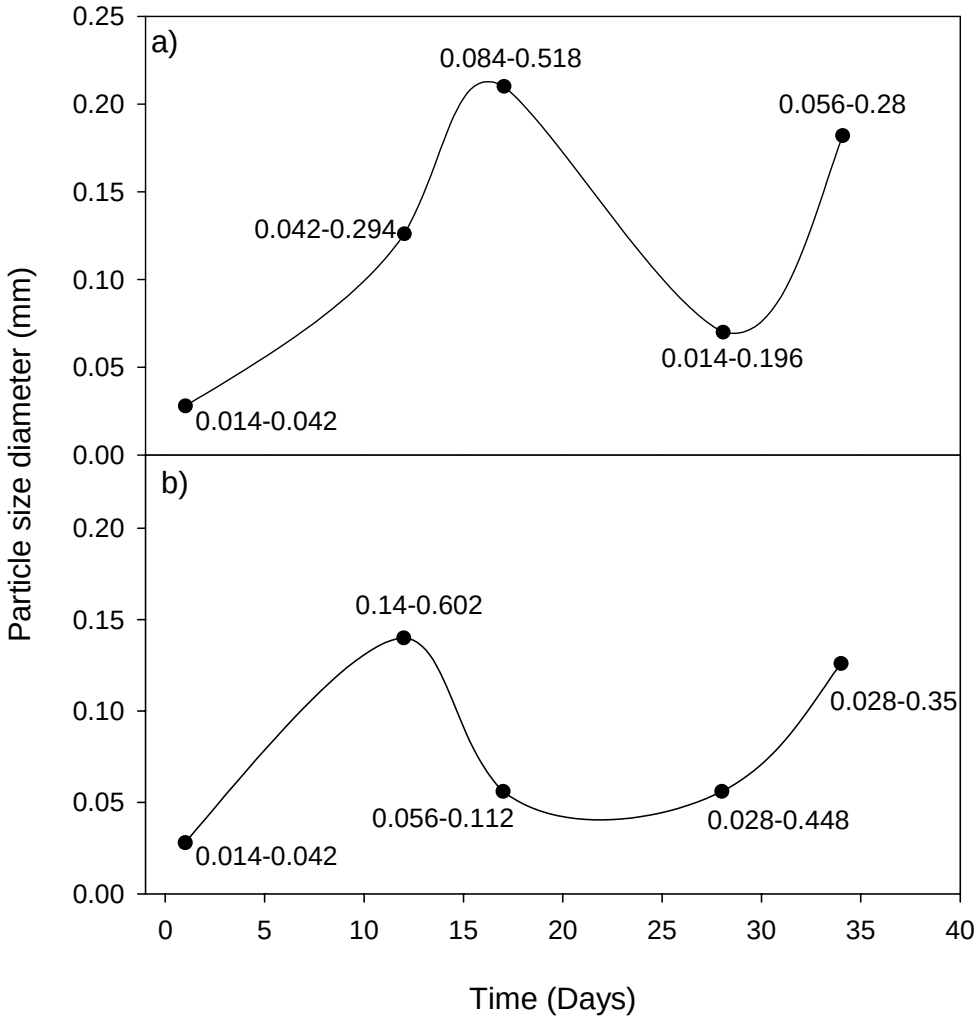

Figure E.1. The changes in the median particle diameters of the cultures in a) R1 (10 mL air/min), b) R2 (60 mL air/min) (ranges given for each datum indicate the minimum and maximum particle sizes observed at that analysis).

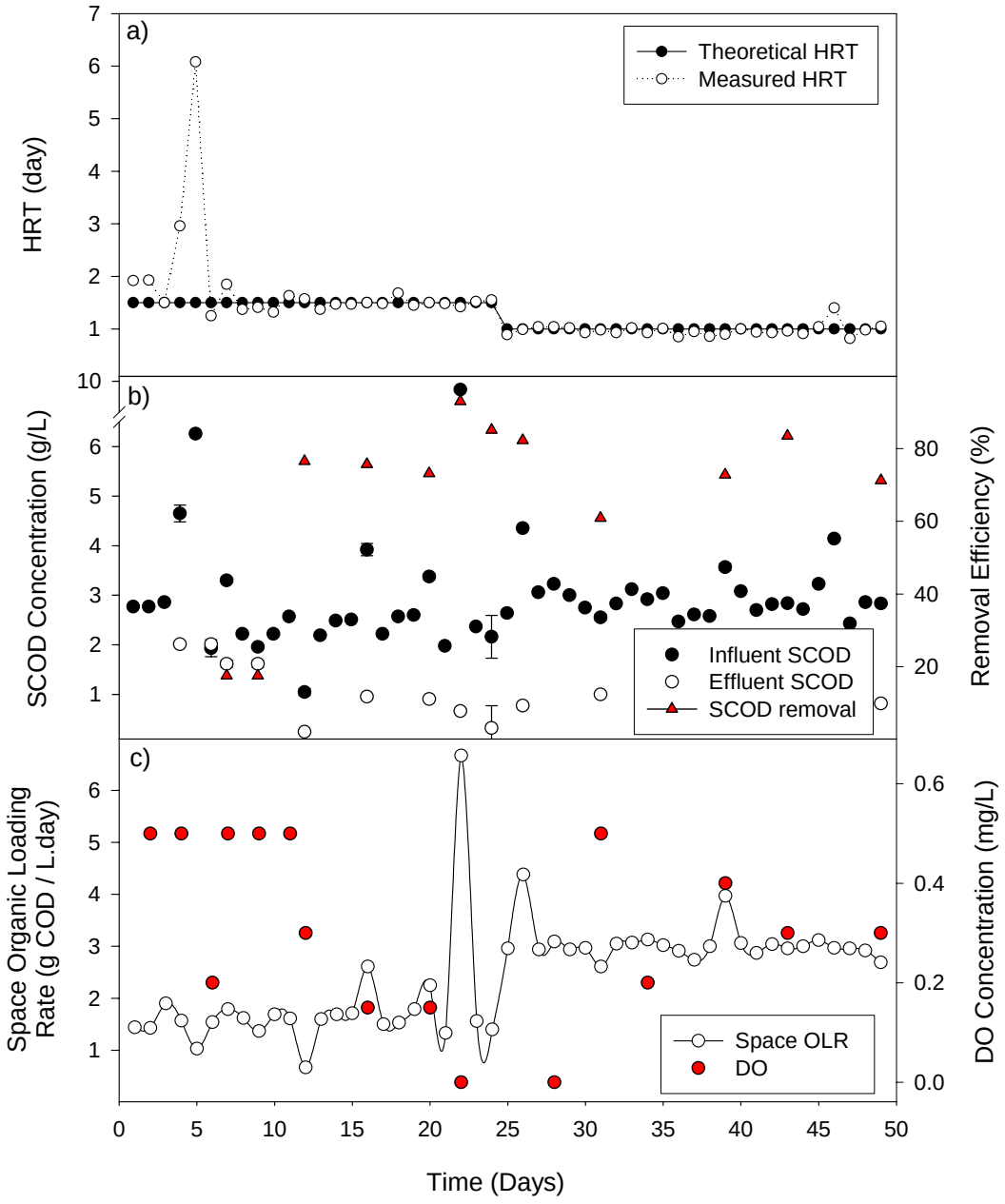

Figure E.2. The operational conditions and results of the experiments conducted with R1 (in terms of HRT, COD/DO concentrations, SCOD removal efficiency and SOLR).

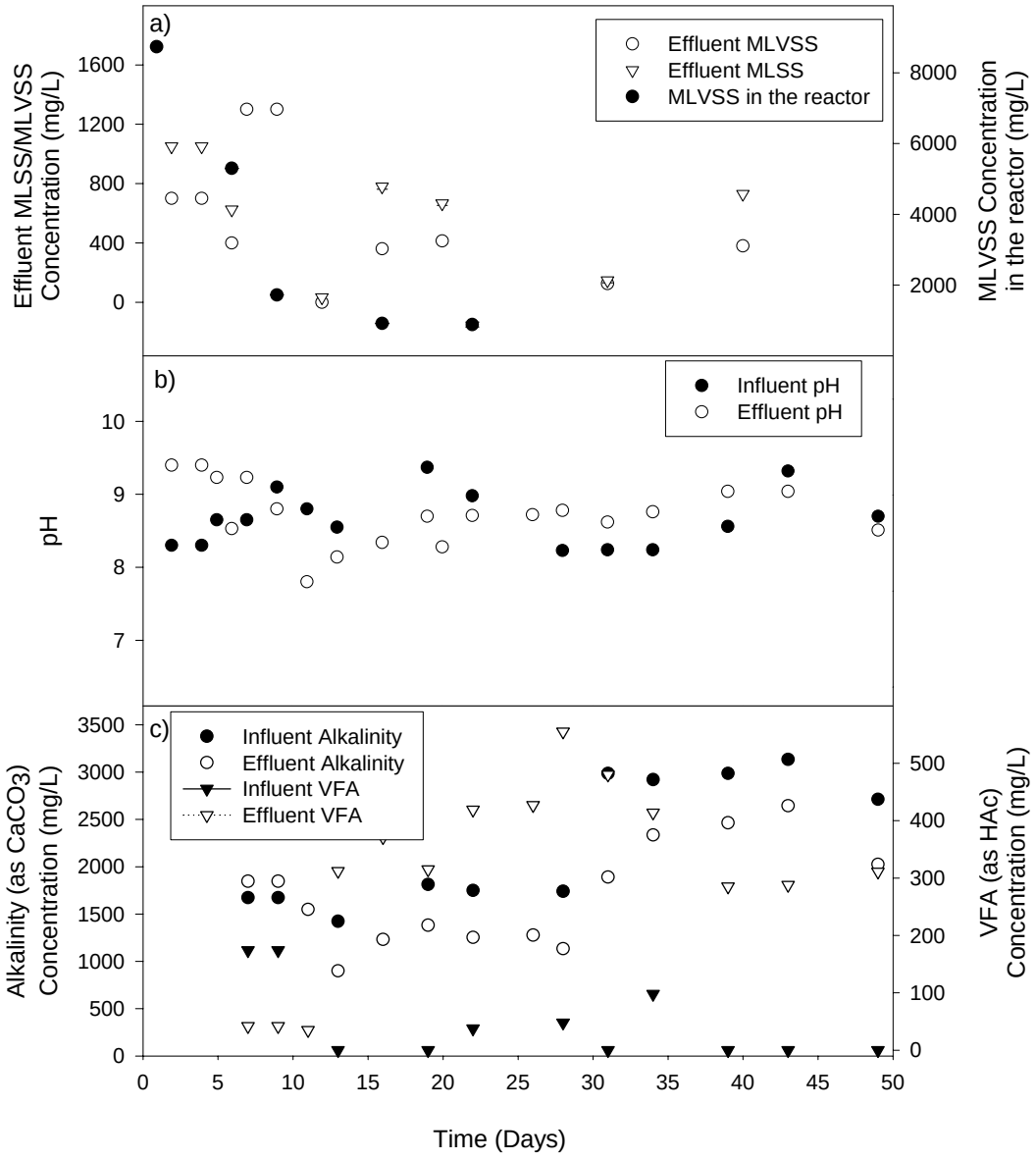

Figure E.3. The operational conditions and results of the experiments conducted with R1 (in terms of MLSS / MLVSS, alkalinity, VFA concentrations and pH).

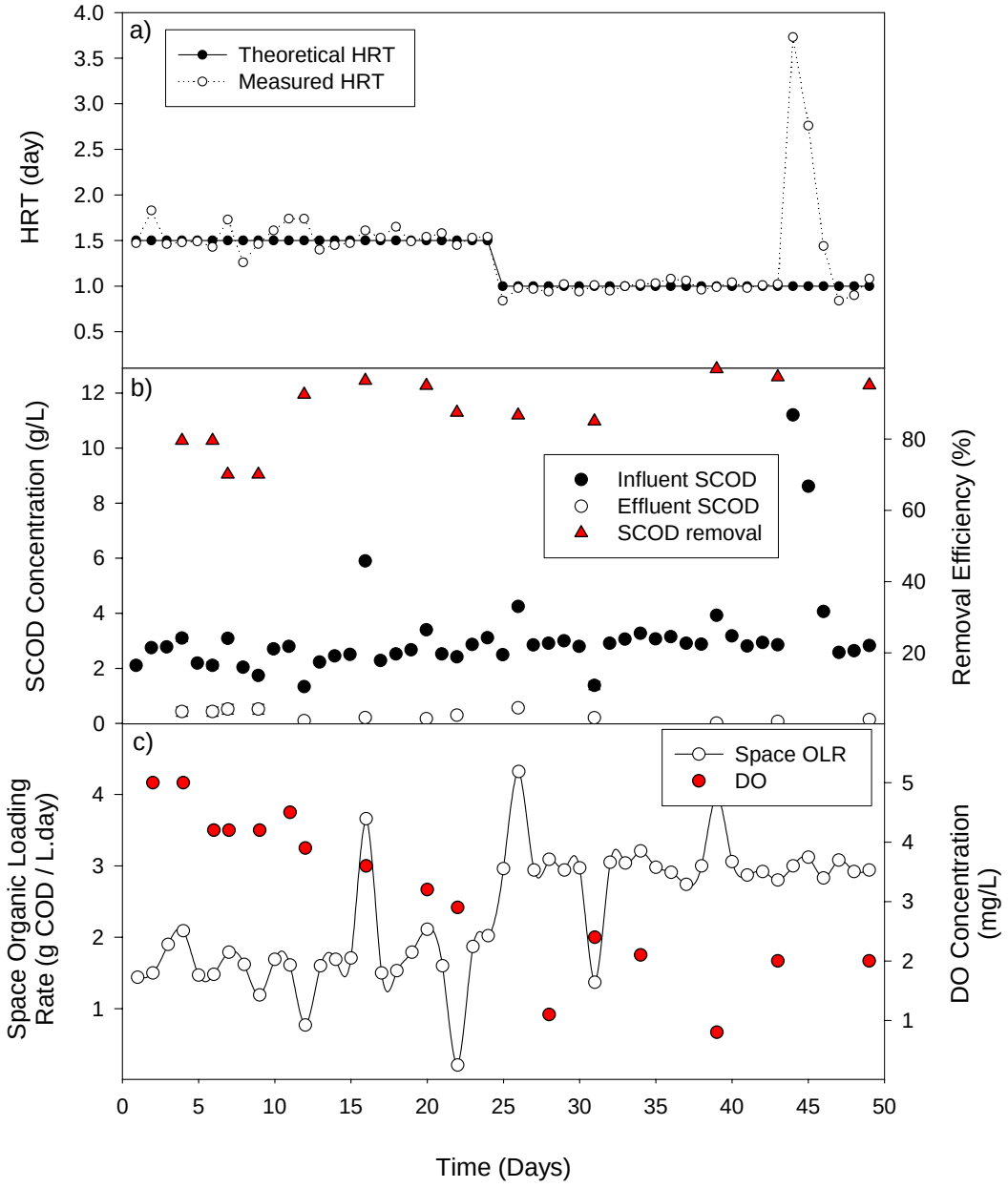

Figure E.4. The operational conditions and results of the experiments conducted with R2 (in terms of HRT, COD/DO concentrations, SCOD removal efficiency and SOLR).

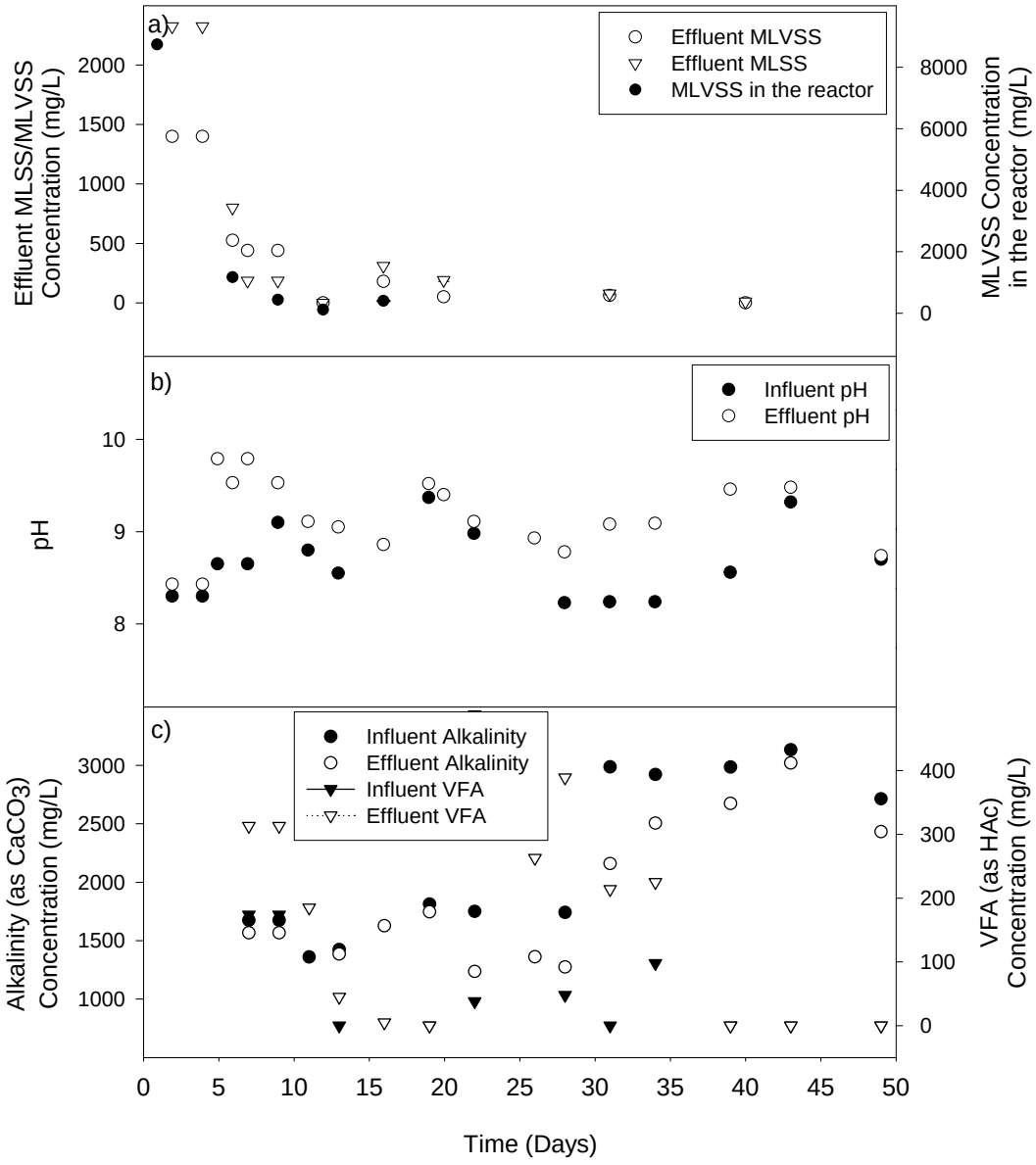

Figure E.5. The operational conditions and results of the experiments conducted with R2 (in terms of MLSS / MLVSS, alkalinity, VFA concentrations and pH).

APPENDIX F

AN EXAMPLE CALCULATION USED TO DETERMINE THE FEASIBILITY OF USING COMBINED CULTURES IN TERMS OF AIR APPLIED TO THE SYSTEM

An example calculation was performed for the USB reactor R2 in study 3, which was aerated 4 hours/day at an air flow rate of 10 mL/min. The calculation given below was based on the treatment performances and the experimental data obtained on Day 359.

<u>The experimental data obtained for R2 (Day 359) :</u>

Volume of wastewater treated = 176 mL/day = 1.76×10^{-4} m^3/day

Influent BOD_5 = 92.5 mg/L

Removal efficiency = 59%

Air flow rate = 10 mL/min

Total volume of air applied per day = 10 mL/min × 4 hours/day × 60 min/hr
= 2400 mL/day = 2.4 L/day
= 2.4×10^{-3} m^3/day

Total mass of BOD_5 applied per day = 176 mL/day × 92.5 mg/L × 10^{-3} L/mL
= 16.28 mg/day = 1.628×10^{-5} kg/day

Total mass of BOD_5 removed per day = $0.59 \times 1.628 \times 10^{-5}$ kg/day
= 9.605×10^{-6} kg/day

$$\frac{Volume\ of\ air\ applied}{Mass\ of\ BOD_5\ applied} = \frac{2.4\times10^{-3}\ m^3\ /\ day}{1.628\times10^{-5}\ kg\ /\ day} = 147\ m^3\ air\ /\ kg\ BOD_5\ applied$$

$$\frac{Volume\ of\ air\ applied}{Mass\ of\ BOD_5\ removed} = \frac{2.4\times10^{-3}\ m^3\ /\ day}{9.605\times10^{-6}\ kg\ /\ day} = 250\ m^3\ air\ /\ kg\ BOD_5\ removed$$

$$\frac{Volume\ of\ air\ applied}{Volume\ of\ wastewater\ treated} = \frac{2.4\times10^{-3}\ m^3\ /\ day}{1.76\times10^{-4}\ m^3\ /\ day} = 13.6\ m^3\ air\ /\ m^3\ wastewater$$

Similar calculations were carried out for each reactor studied. Calculations were based on the daily data (i.e. influent BOD_5 concentration, BOD_5 removal efficiency, and wastewater and air flow rates) of each USB reactor obtained during municipal wastewater application period. The values for volume of air applied per kg BOD_5 applied and removed, and per volume of wastewater treated were interpreted in terms of ranges and average values.

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SELECTED PUBLICATIONS

1. Erguder T.H. and Demirer G.N. “Granulation of a mixture of a suspended anaerobic and aerobic cultures under alternating anaerobic / microaerobic / aerobic conditions: A preliminary study”. J. of Chem. Technol. Biotechnol., in press.

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4. Erguder T.H., Guven E. and Demirer G.N. “The inhibitory effects of lindane in batch and upflow anaerobic sludge blanket (UASB) reactors”. Chemosphere, 50, 165-169 (2003).

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10. Uzal N., Erguder T.H., Tezel U., Imamoglu I. and Demirer G.N. “The application of natural zeolites in environmental pollution control and an example application for Cu(II) removal”, 2th National Environmental Pollution Control Symposium. October 22-24, Ankara, Turkey (2003).

11. Erguder T.H., Guven E. and Demirer G.N. “The inhibitory effect and removal of an organochlorine insecticide, dieldrin, in anaerobic treatment systems” 4th National Environmental Engineering Symposium, November 7-10, Mersin, Turkey (2001).

12. Erguder T.H., Guven E. and Demirer G.N. “The inhibitory effects of two organochlorine insecticides; lindane and dieldrin on anaerobic cultures”. 9th World Congress on Anaerobic Digestion, September 2-6, Antwerpen, Belgium (2001).

13. Erguder T.H., Demirer G.N. "Anaerobic treatment of olive mill wastes (OMWs) in batch reactors". 10th International Symposium on Environmental Pollution and Its Impact on Life in Mediterranean Region, MESAEP, October 2-6, Alicante, Spain (1999).