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EFFECT OF REDOX CONDITIONS IN THE DEGRADATION OF (*RS*)-MCPP DURING BIOLOGICAL TREATMENT OF SYNTHETIC WASTEWATER IN AN ANAEROBIC MEMBRANE BIOREACTOR (AMBr)

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ABSTRACT

The aim of this research is to investigate the effect of mecoprop (*RS*-MCPP) degradation in an anaerobic membrane bioreactor (AMBr) under a range of redox conditions (methanogenic, nitrate reducing and sulphate reducing). A method was developed for the analysis of (*RS*)-MCPP in aqueous form using High Performance Liquid Chromatography (HPLC).

The study was carried out in five phases. Initially, **Phase I** investigated the effect of elevated *(RS)*-MCPP on AMBr under methanogenic conditions. In this phase, increasing concentrations of (*RS*)-MCPP (5 – 200 $mg.L^{-1}$) promoted a progressive increases in the (*RS*)-MCPP substrate utilisation rate (SUR) (0.15 to 3.19 $\mu g.mgVSS^{-1}.d^{-1}$). However, the COD removal efficiency decreased gradually from 98 % to 94 % as the (*RS*)-MCPP concentration increased in the feed from 50 – 200 $mg.L^{-1}$, respectively.

Phase II investigated the effect of denitrifying conditions on *(RS)*-MCPP degradation. Results showed that (*RS*)-MCPP removal efficiency and (*RS*)-MCPP SUR improved from 2 – 47 % and 0.5 to 60.7 $\mu g.mgVSS^{-1}.d^{-1}$, respectively as the COD/N-NO_3^{-1} ratio and OLR were reduced from 250 to 0.2 and 1.51 to 0.07 $kgCOD.m^{-3}.d^{-1}$, respectively.

Phase III investigated the effect of sulphate-reducing conditions on *(RS)*-MCPP degradation. Results showed that sulphidogenic conditions were less effective than nitrate reducing conditions with an average (*RS*)-MCPP removal and (*RS*)-MCPP SUR at 20(±10.7) % and 16.4 $\mu g.mgVSS^{-1}.d^{-1}$, respectively.

Phase IV investigated the effect of HRT on the kinetics of (*RS*)-MCPP degradation. The robustness and stability of AMBr was also investigated in response to hydraulic shock loads on the biomass imposed. Results showed that the AMBr is stable to a large transient hydraulic shock loads, and it recovered rapidly to baseline performance after the hydraulic shock load had ended. High (*RS*)-MCPP degradation (up to 75 %) and utilisation rates (43 $\mu g.mgVSS^{-1}.d^{-1}$) were observed at long HRT (16.9 days).

Phase V was a confirmatory experiment study to investigate the possible effects of trace quantities of oxygen that had been present in the reactor headspace and the effluent tank during Phase IV. This confirmed that the system performance towards (*RS*)-MCPP biodegradation had not been affected by trace oxygen levels when comparison are made.

The molecular-based method, fluorescent *in situ* hybridisation (FISH) was used for the direct identification and enumeration of microbial communities in the AMBr sludge. In general, FISH results showed populations were dominated by methanogenic archaea in methanogenic phase (Phase I and IV) comprising between 55-66 % of the DAPI count. However, as anoxic condition were introduced in Phase II and III (nitrate reducing and sulphate reducing), the results identified a definite population shift, with the predominance of eubacteria (70-86 % of the DAPI count). The two methanogenic genera detected were *Methanosaeta* and *Methanosarcina*. Of these two genera, the former was dominant in the reactor accounting for 57-100 % of the total methanogenic archaea. Furthermore, a comparison of (*RS*)-MCPP utilisation rates in each phase of the research revealed wide ranges (*RS*)-MCPP degradation

efficiency and (*RS*)-MCPP SUR, however, although changes were observed in the composition of the bacterial population, no direct linkage could be made with any of the individual groups identified by FISH.

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LIST OF PUBLICATION

Ali Yuzir and P.J.Sallis, 2004. Treatment of acidic herbicides wastewater using Anaerobic Membrane Bioreactor (AMBr) approach. In: *Postgraduate Research Conference*, School Of Civil Engineering and Geosciences, 25th June 2004, University of Newcastle upon Tyne, UK. (Poster Presentations)

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combination with a more specific probe (MX825) labelled with Cy5 (emits as blue). Only cells fluorescing magenta (D) would be identified as MX825.

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ABBREVIATIONS

AMBr	Anaerobic Membrane Bioreactor
APHA	American Public Health Association
BOD	Biological Oxygen Demand
CLSM	Confocal Scanning Laer Microscope
COD	Chemical Oxygen Demand
DAPI	4', 6-diamido-2-phenylindole
FISH	Fluorescent *in situ* Hybridisation
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
MLSS	Mixed Liquor Suspended Solid
MLVSS	Mixed Liquor Volatile Suspended Solid
MPA	Methane Producing Archaea
MPB	Methane Producing Bacteria
OLR	Organic Loading Rate
rDNA	Ribosomal Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SRB	Sulphate Reducing Bacteria
SRT	Solid Retention Time
SS	Suspended Solid
STP	Standard Temperature and Pressure / Sewage Treatment Plant
SUR	Substrate Utilisation Rate
TOC	Total Organic Carbon
VFA	Volatile Fatty Acids

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

The most basic role provided by wastewater treatment plants is to reduce and protect the environment from contaminant discharges to the receiving water. Environmental and public health engineers play an important role in the field of wastewater treatment by evaluating possible solutions in the design, build and operation of treatment systems. Wastewater treatment processes can be divided into three major types: physical, chemical and biological. Of these, biological processes are the most commonly used especially for municipal and industrial effluents. All treatment plants use energy, and since the energy crisis in the early seventies, energy reduction has attracted increasing interest from academia and practitioners in the field of environmental engineering to find new ways of reducing energy costs. One such option is to switch from conventional aerobic treatment plants to plants utilising anaerobic technologies.

Many researchers in the field of environmental engineering have carried out extensive studies on anaerobic digester design. It is widely believed that anaerobic treatment has many advantages over aerobic processes; in particular the useful methane by-product from the process could offset energy costs. More recently, research has shown new initiatives that improve the anaerobic treatment processes by combining it with membrane separation; this was first mentioned by Smith et al., in 1969. This fast growing technology, known as an

Anaerobic Membrane Bioreactor (AMBr) offers several positive features including reduced plant size, production of a very high quality final effluent, lower energy requirements for maintenance, compactness of equipment, faster start-up and plant automation (Fakhrul-Razi & Noor, 1999).

In the AMBr, solids retention time (SRT), which is related to the growth rate of microorganisms in the biomass, is completely independent of hydraulic retention time (HRT) by means of membrane separation. Therefore, a high SRT can be achieved in AMBr, which favours the slow growth rate of anaerobic microorganisms, whilst maintaining a short HRT, which is paramount to reducing the cost of the treatment plant; hence this is the preferred process compared to conventional anaerobic digesters. Studies on anaerobic membrane process have showed the effectiveness of treating high strength wastewaters such as wine distillery effluents (Ross et al., 1990), palm oil mill effluent (Fakhrul-Razi & Noor, 1999), dairy effluent (Li et al., 1985), hospital wastewater (Wen et al., 2003) and domestic wastewater (Wen et al., 1999).

Mecoprop (2-(2-methyl-4-chlrophenoxy)-propionic acid) or (*RS*)-MCPP is a toxic compound and has been widely used as herbicide for agriculture and horticulture purposes, it has a high water solubility at 734 $mg.L^{-1}$ at 25°C (Tomlin, 1997) and a relatively low sorption to soil (Lipthay et al., 2006) making it susceptible to surface runoff to nearby streams, leaching to underlying groundwater aquifers and entering sewerage systems and surface water. It falls within List 1 of the Groundwater Regulations 1998 (Statutory Instrument 1998

No. 2746) and its maximum concentration for potable water set by European Union under the 1980 EC Directive on drinking water (80/778/EEC) is 0.1 μgL^{-}.

(*RS*)-MCPP is in a same group as MCPA, 2,4-D and MCPB which are referred to as chlorophenoxyalkanoic acid compounds. In the UK, (*RS*)-MCPP is most commonly used as a selective herbicide for the control of broadleaved weeds in cereal crops, predominantly due to its use with the production of winter cereals, as herbicide applications being made in autumn and spring (Fletcher et al., 2004) and was detected up to 8 $\mu g.L^{-1}$ (*RS*)-MCPP in a public supply borehole at Etton, 2.5 km east of landfills near Peterborough (Williams et al., 2003) as a result of disposal either directly or from grass cuttings which then leached to groundwater.

Its application is not restricted to agriculture and horticulture, it is also used in construction as an algicide in paints and coatings and roof protection agents. According to Bucheli et al., (1998), (*RS*)-MCPP was detected at concentration levels up to 500 $\mu g.L^{-1}$ in the runoff from roofs that have been treated with the treatment agent Preventol B2, a bi-ester of (*RS*)-MCPP. Furthermore, they suggested that the contamination load from the roof runoff was in the same order of magnitude as the load generated by agricultural applications. In 1998, 300 t/year of (*RS*)-MCPP was used as a roof protection agent in Switzerland alone (Bucheli et al., 1998).

(*RS*)-MCPP is considered one of the key indicators of pollution as it is frequently detected in municipal wastewater, surface water, landfill leachate and groundwater in the range of nano- to micro-grams per litre (Bucheli et al., 1998; Petrovic & Larsson-Kovach, 1996; Zipper, et al., 1998; Fletcher et al., 2004). However, these figures are probably higher in areas affected by point source contamination (Figure 1.1) where concentrations may reach milligrams per litre (Gerecke et al, 2002). (*RS*)-MCPP has various toxicity effects on human, animals and aquatic organisms at a level of: 650 $mg.kg^{-1}$ for mice (Meister, 1992; Thomson, 1982; Budavari, 1989), 124 $mg.L^{-1}$ for rainbow trout (USEPA, 1986) and 237 $mg.L^{-1}$ for algae (AHMARKS, 2005). It has also been reported that overexposure to herbicide can increase neurological symptoms and psychomotor dysfunction (Kamel & Hoppin, 2004).

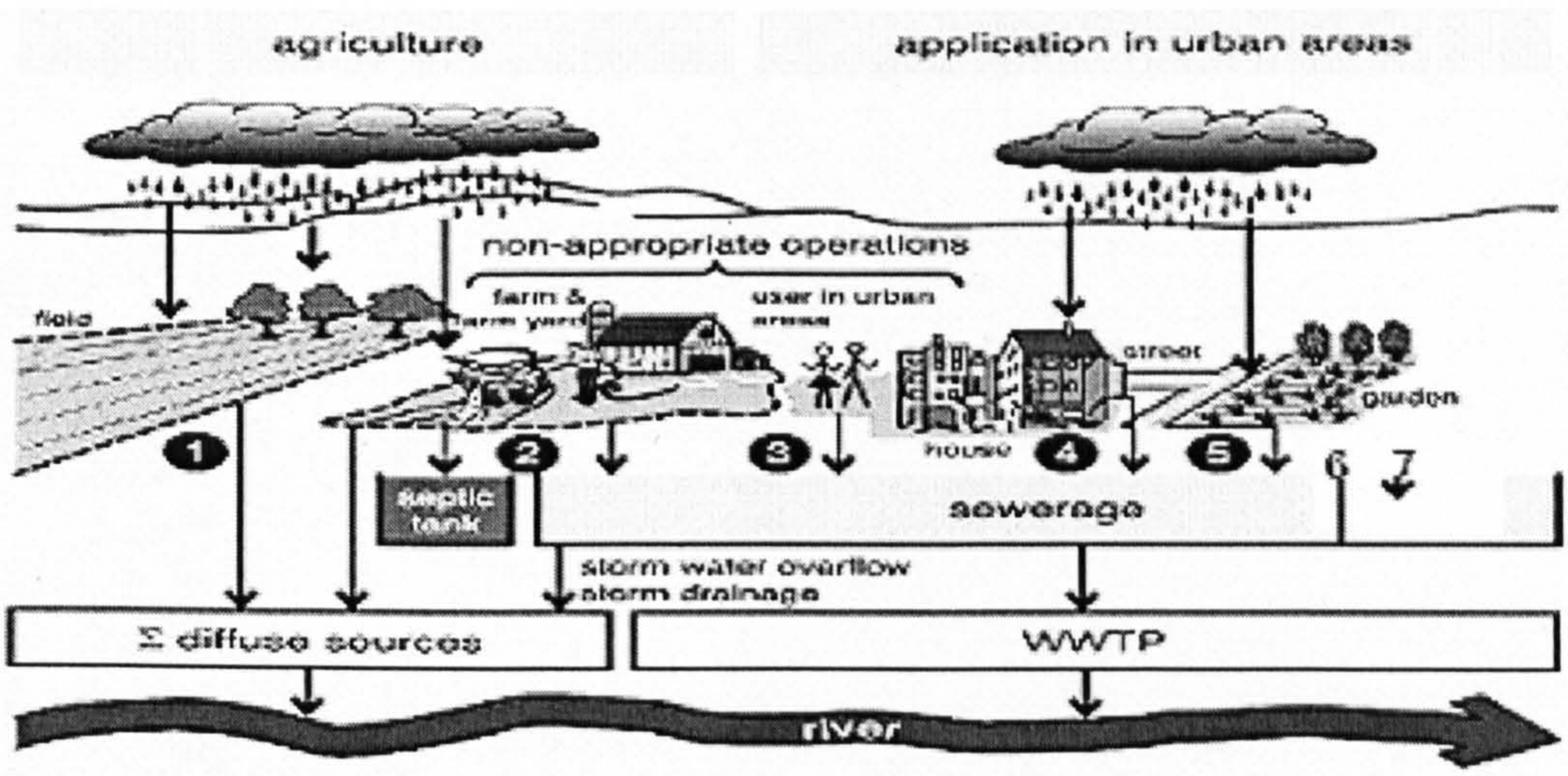


Figure 1.1: Applications and major pathways of herbicide transport into surface waters (Taken from Gerecke et al, 2002).

Figure 1.1 shows possible pathways for the transportation of (*RS*)-MCPP into river systems. Agricultural applications, e.g.. spray drift, may contaminate rivers

directly or cause toxicity to aquatic systems through diffuse surface or subsurface hydrological pathways during rain events (source 1). Moreover, improper operations in farm such as washing, measuring and filling sprayer equipment may cause inputs to sewers (source 2). Similarly in urban areas, application such as non-appropriate operations (source 3), application of Preventol B2 in building material (source 4) and application on lawns and streets (source 5) could contaminate sewers during rain. In the production site, effluent from manufacturing site enters wastewater treatment plant (source 6). Another major pathway is via landfills since herbicides are often disposed of in landfills (source 7).

Although the fate and degradation of (*RS*)-MCPP has been investigated (Toräng, et al., 2003, Williams, et al., 2003, Larsen and Aamand, 2001 and Tuxen et al., 2006) this work has been carried out under aerobic conditions, and only a very limited number of studies have been reported on its degradation under anaerobic conditions, most of this focusing mainly on groundwater, aquifer and landfill leachates (Baun et al., 2003, Harrison et al., 2003 and Larsen and Aamand, 2001), and no research has been dedicated solely to the anaerobic recalcitrance of (*RS*)-MCPP during the anaerobic treatment of wastewaters.

Thus, a combination of anaerobic digestion and membrane separation technology, referred to as an Anaerobic Membrane Bioreactor (AMBr), would give a significant contribution to our understanding of the anaerobic degradation (*RS*)-MCPP in wastewaters containing high levels of this compound, and the

potential of this and other anaerobic processes to treat effluents from (*RS*)-MCPP manufacturing sites, landfill leachate and other highly contaminated sources.

An additional factor which may affect the efficiency of (*RS*)-MCPP degradation during anaerobic treatment is the possible presence of alternative terminal electron acceptors, such as nitrate and sulphate, which may be present at significant concentrations in the wastewaters from herbicide manufacturing plants. These will have a bearing on the redox conditions and alter the composition of the microbial populations in the reactor, both of which are likely to have an impact on the fate of (*RS*)-MCPP during treatment.

This study aims to show the feasibility of using a laboratory-scale AMBr system to treat a synthetic herbicidal wastewater containing (*RS*)-MCPP, under different electron acceptor conditions, by supplementing nitrate and sulphate in the reactor feed in order to achieve different redox conditions, i.e. methanogenic, nitrate-reducing and sulphate-reducing.

Due to time and resource limitations, it was decided to focus on investigating a wide range of operating conditions and to run each set of experimental conditions over extended periods to allow adequate time for acclimation of the biomass to occur. This meant that a detailed characterisation of breakdown intermediates and the metabolic pathway could not be undertaken. Equally, analysis of the microbial communities was limited to following key genera by FISH and the research did not attempt to distinguish which microorganisms were responsible for (*RS*)-MCPP, or characterise species-level changes using DGGE.

The research was performed under five separate phases carried out sequentially using the same reactor and MLVSS. **Phase I** investigated the effect of elevated (*RS*)-MCPP concentration on AMBr under methanogenic conditions. In contrast, **Phase II and III** investigated (*RS*)-MCPP degradation under nitrate and sulphate-reducing conditions respectively. Towards the end of the project, **Phase IV & V** investigated the effect of step increases in HRT on (*RS*)-MCPP degradation under methanogenic conditions. **Phase V** was a control experiment for Phase IV which further investigated the possible effect of trace dissolved oxygen concentration on (*RS*)-MCPP degradation.

1.2 THESIS ORGANISATION

This thesis is organised into ten chapters as follows.

Chapter 2 provides an overview of relevant literature and covers basic principles of anaerobic digestion, methanogenesis, sulphate reduction and denitrification processes. To give the reader a clearer view of the Anaerobic Membrane Bioreactor (AMBr) design, a brief review of membrane types and processes is given. This chapter also introduces (*RS*)-MCPP in relation to its chemistry, physico-chemical properties, application, contamination pathways and biodegradation factors. Towards the end of the chapter, the molecular analysis techniques used to analyse microbial communities in reactor sludge; DAPI and FISH are addressed.

Chapter 3 outlines the aim and scope of the study.

Chapter 4 describes the laboratory equipment. Wastewater feed, nutrients and sludge used in this study, defining reactor start-up procedure, operation and the

standard analyses used throughout the study. A brief description on method development for (*RS*)-MCPP detection by High Performance Liquid Chromatography (HPLC) is also presented.

Chapters 5, 6, 7, 8 present the results of the three main AMBr studies treating synthetic wastewater containing (*RS*)-MCPP under different electron accepting conditions by adding specific terminal electron acceptors, nitrate and sulphate, into the reactor feed

Chapter 9 is devoted to the molecular analysis of microbial communities growing in the AMBr sludge in the above experiments using Fluorescent *in situ* Hybridisation (FISH).

The conclusions from this research are given in **Chapter 10,** stating specific achievements, problems and recommendations. General conclusions are also drawn from the experience gained during this work, the wider implication of the results and some recommendations for future research.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

This chapter is divided into eleven sections. The first (Section 2.2) contains a review of anaerobic digestion emphasising the principles (mechanism and microbiology) and the operational factors affecting anaerobic digestion. Sulphate-reducing and denitrification processes are reviewed in Section 2.3 and 2.4. Furthermore, the microbiological aspects, operational factors and interaction between these processes and methanogens in an anaerobic digester are also discussed.

A review of the reactor used in this study, Anaerobic Membrane Bioreactor, (AMBr) with a brief explanation on membrane types and processes are described in Section 2.5 and 2.6. An overview of herbicides in environment and an introduction to the herbicide used in the study, (*RS*)-MCPP and its contamination pathways are given in Section 2.7 and 2.8. Further review on (*RS*)-MCPP biodegradation processes and possible influencing factors on the degradation process are given briefly in Section 2.9. To further investigate the bacterial population in the AMBr, the molecular techniques, a brief introduction to 4',6-diamido-2-phenylindole (DAPI) staining, fluorescent *in situ* hybridization (FISH) and confocal scanning laser microscope (CLSM) are described in Section 2.10. Finally, the literature review is summarised in Section 2.11.

2.2 ANAEROBIC BIOLOGICAL DIGESTION

Anaerobic treatment is the stepwise conversion of large molecules of organic compounds into methane and carbon dioxide by microbial consortia in the absence of free oxygen. There are three main stages to the anaerobic digestion process involved: hydrolysis, acid formation and methane formation, each being carried out by a separate group of bacteria.

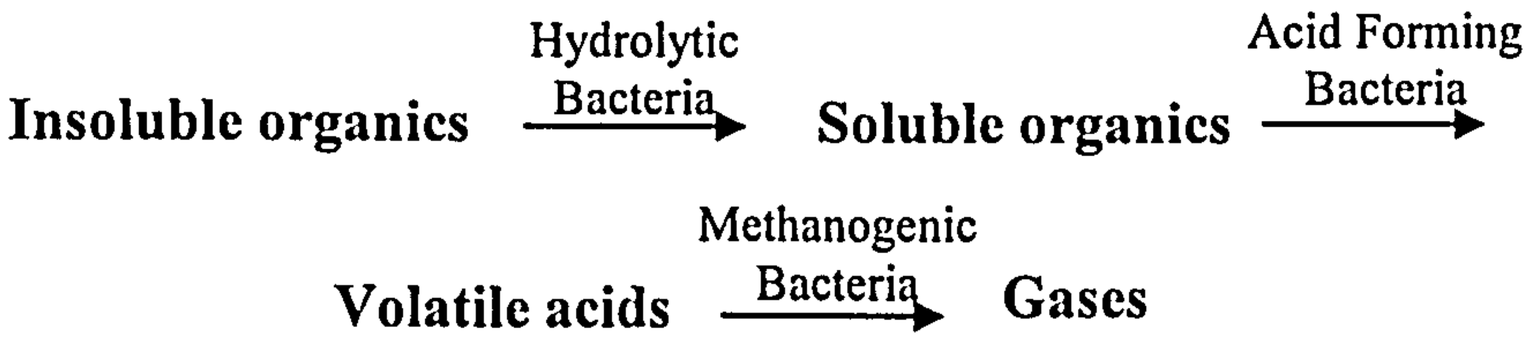


Figure 2.1: Simple schematic stages of the anaerobic digestion process.

In the first stage, complex organic matter, such as proteins, carbohydrates and lipids, are hydrolysed to smaller and more soluble forms of organic matter; amino acids, monosaccharides and long-chain fatty acids. These simple carbohydrates and acids are then converted to organic acids and hydrogen as the dominant intermediate products in the second stage (Rittmann and McCarty, 2001). In the final stage, these organic acids are then converted to gases, mainly methane and carbon dioxide (Gray, 2004). A reaction sequence with COD percentage channelled through different metabolic rates and designing the predominant microorganisms responsible for each step in the anaerobic process is shown in Figure 2.2.

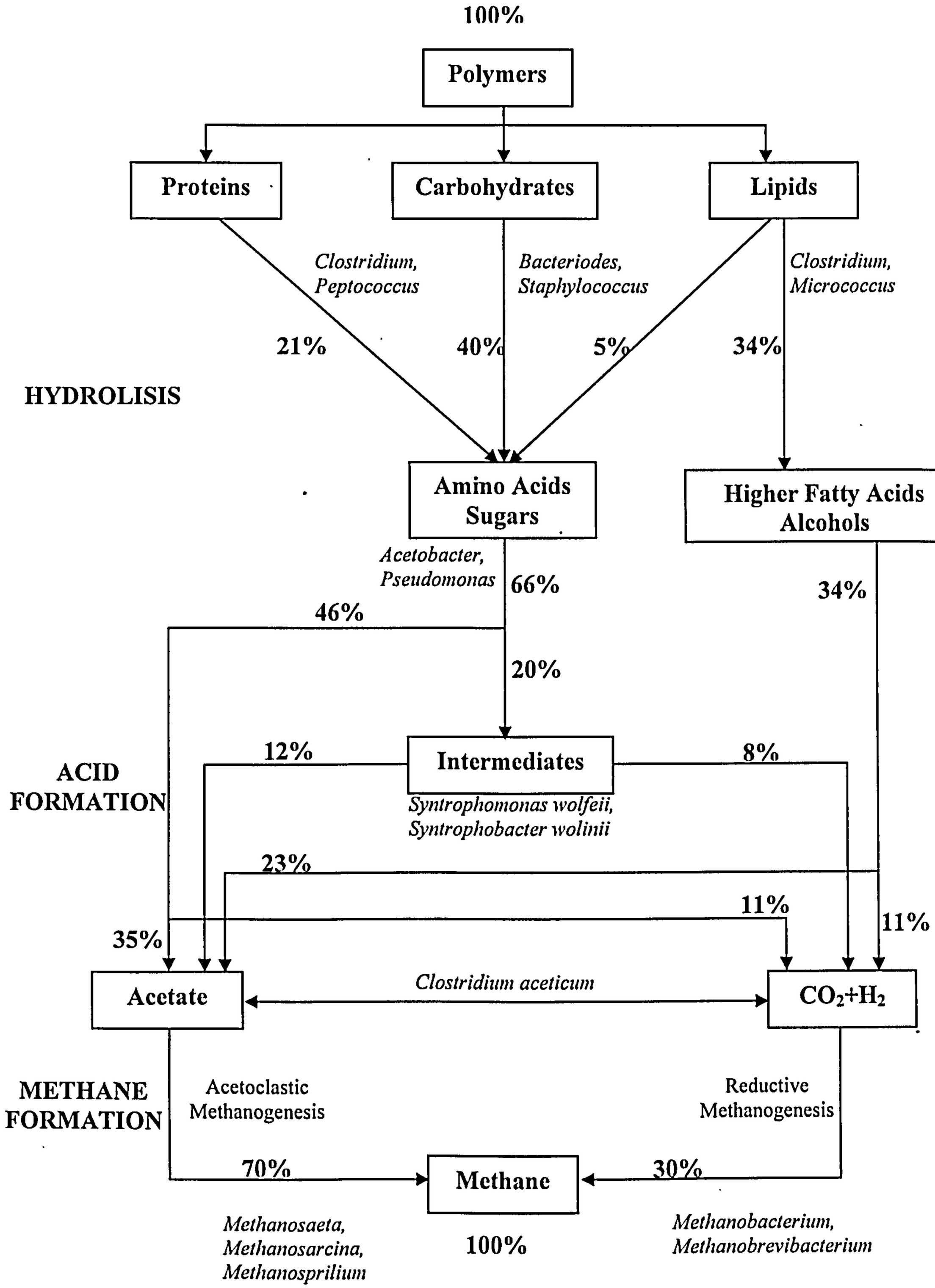


Figure 2.2: Stepwise reactions for the anaerobic digestion of polymeric materials with COD flow and the microorganisms predominantly responsible for each step (adapted from Gujer and Zehnder, 1983).

2.2.1 Microbiology of the Anaerobic Process

Anaerobic digestion is a complex process involving many groups of bacteria and several intermediate steps. It requires the presence of a diverse and closely dependent group of bacteria to bring about the complete conversion of a complex mixture of substrates to methane gas (McCarty and Smith, 1986). There are six distinct processes that may be identified in an anaerobic digester as proposed by van Haandel and Lettinga (1995):

i. Hydrolysis of complex organic polymers such as carbohydrates, proteins and fats,
ii. Fermentation of amino acids and sugars,
iii. Anaerobic oxidation of long chain fatty acids and alcohols,
iv. Anaerobic oxidation of intermediary products such as volatile fatty acids, except acetate,
v. Conversion of acetate to methane,
vi. Conversion of hydrogen to methane.

The first stage of the anaerobic process involves hydrolytic bacteria (hydrolysis), followed by acid forming bacteria (acid formation) in the second stage. The latter consists of both acidogenic (organic acid forming) and acetogenic (acetate forming) bacteria. The final metabolic steps are completed by the methanogenic bacteria (methanogenesis) which consume the end products from the second stage and convert them to the final end-products namely methane and carbon dioxide. This stage involves two distinct groups, those utilising acetate and those utilising hydrogen and carbon dioxide. The following sections will outline these general classes of metabolism in more detail.

2.2.1.1 Hydrolysis Reactions

Hydrolysis represent one of the most important steps in anaerobic digestion: to break down complex organic polymers such as carbohydrates, proteins and fats into much simpler and smaller substrates which are then accessible to further degradation processes. This is carried out by means of extracellular hydrolytic enzymes such as cellulase, amylase, lipase, pectinase, chitinase, protease etc. (Kaseng et al., 1992). In this process, complex insoluble particulate matter is converted into dissolved compounds with a lower molecular weight. It requires the mediation of exo-enzymes that are excreted by fermentative hydrolytic bacteria such as *Clostridium*, *Micrococcus, Peptococcus* and *Staphyloccus*, which are commonly facultative in nature so that they have the ability to breakdown those organic solids in both the presence and absence of oxygen. Being extracellular these enzymes are able to access large substrate molecules that are incapable of crossing the bacterial cell wall due to their size.

The rate of hydrolysis is influenced by many factors including pH, cell age, wastewater content and the hydrolysis rate of lipids, which in particular becomes very low below 20°C. In practice, the hydrolysis rate can often be limiting for the overall rate of anaerobic digestion (Lettinga, 1995) particularly for complex insoluble polymers. Consequently, if large quantities of solids or particulates are present in the feed stream, the degradability also decreases due to poor solubilisation of these solids by hydrolytic enzymes, resulting in a rate-limiting step for the process.

2.2.1.2 Acidogenesis

Acidogenesis is the second stage of ànaerobic digestion. It is a fermentation process for organic matter, such as amino acids, sugars, fatty acids and alcohols, produced from the hydrolysis reactions. The sugars, long-chain fatty acids and amino acids resulting from hydrolysis are used as substrates by fermentative organisms or by anaerobic oxidisers (Gujer and Zehnder, 1983). There are many different fermentative genera and species in this stage of the process, among them are *Clostridium, Bacteroides, Ruminococcus, Butribacterium, Propionibacterium, Eubacterium, Lactobacillus*, *Streptococcus, Pseudomonas, Desulfobacter, Micrococcus, Bacillus* and *Escherichia*. Typical cell counts of acidogens in anaerobic digesters range from 10^6–10^8 per ml (Archer and Kirsop, 1990). Acidogenesis is usually the fastest step during the anaerobic conversion of complex organic matter in liquid phase digestion (Mosey and Fernandes, 1989).

Under low hydrogen concentrations, the main degradation pathway is through acetate which is the main substrate for methanogens because hydrogen is quickly removed by other hydrogen consuming bacteria. This degradation pathway gives higher energy yield for acidogens and also provides substrates for methanogenic microorganisms. Many of the fermentation products cannot be utilised directly as substrates by the methanogenic microorganisms but must be degraded further by the obligate hydrogen producing bacteria (see below) in a process that is known as acetogenesis. This is an important step in anaerobic sewage treatment, as dissolved oxygen might otherwise become toxic to obligate anaerobic organisms such as the methanogens (Lettinga, 1995).

2.2.1.3 Acetogenesis

Acetogenic bacteria such as *Syntrobacter wolinii* and *Syntrophobacter wolfeii* oxidize the volatile fatty acids and alcohols into acetate, hydrogen and carbon dioxide which are the only substrates that can be metabolised efficiently by the methanogens during the final stage of anaerobic digestion. The bacteria are classified into two main groups on the basis of their metabolism. The first group is referred to as the obligate hydrogen-producing acetogens (OHPA), also called proton-reducing acetogens, which produces acetic acid, carbon dioxide and hydrogen from the major fatty acid intermediates including propionate and butyrate, alcohols and other higher fatty acids such as valerate, isovalerate stearate, palmitate and myrisyate via the process of β-oxidation. OHPA species are particularly important in the β-oxidation of longer-chain fatty acids arising from lipid hydrolysis and are also involved in the anaerobic degradation of aromatic compounds.

The second group of acetogenic bacteria is the homoacetogenic bacteria. These bacteria are strictly anaerobic microorganisms catalysing the formation of acetate from hydrogen and carbon dioxide. Homoacetogenic bacteria are known in the genera *Acetobacterium, Acetoanerobium, Acetogenium, Butribacterium, Clostridium* and *Pelobacter*. They also participate in the interspecies hydrogen transfer process, which maintains the low hydrogen concentrations required by the OHPA. However, their importance in this respect, relative to that of the methanogens, is still not clear although the number of homoacetogenic bacteria in anaerobic digesters is considerably lower, at around $1x10^5$ per ml (Torein and

Hattingh, 1969) than that of the methanogens, suggesting a relatively minor role in hydrogen metabolism.

2.2.1.4 Methanogenesis

The methanogens are the key microorganisms in the production of methane from acetate, hydrogen and carbon dioxide. They are strict anaerobes and form methane gas as the end product of their metabolism. They are known to be a truly distinct group compared to typical bacteria (*Eubacteria*) and are classified in a separate kingdom known as *Archaebacteria.* Without methanogens, the complete anaerobic breakdown of an organic material would not take place due to the accumulation of the end products of the acid-producing bacteria.

Generally, methanogens are most active in the pH range from 6.7 to 8.0 (Hobson and Wheatley, 1993). Therefore, the methanogens will be sensitive in poorly buffered environments to acidification caused by the products of acidogenic and acetogenic bacteria. Only a limited range of substrates can be utilised by methanogens, among these are acetate, hydrogen, carbon dioxide, methanol and formate. As discussed below, methanogens are divided into two groups according to their substrate specificity; acetoclastic methanogens and hydrogen-utilising methanogens. The methanogenic population in sewage sludge can be present at up to 10^8 per ml (Wheatley, 1990).

i. Acetoclastic Methanogens

The most important methanogenic transformations in anaerobic digestion are the acetoclastic reaction and the reduction of carbon dioxide. It has been estimated

from stoichiometric relations that about 70 % of the methane is produced via the acetate pathway (Hobson and Wheatley, 1993). However, very few known species can perform this acetoclastic methane production, whereas nearly all known methanogenic species are able to produce methane from hydrogen and carbon dioxide. Among the species that are capable of utilising acetate (acetoclastic) are *Methanosaeta* (formally known as *Methanothrix*) and *Methanosarcina*. In addition to this acetoclastic activity, *Methanosarcina sp.* is also capable of using methanol, methylamines and sometimes hydrogen and carbon dioxide as growth substrates, while *Methanosaeta sp.* is restricted to growth only on acetate (Schmidt and Ahring, 1996).

The two genera exhibit very different morphologies and growth kinetics. *Methanosarcina sp.* usually grows in aggregates consisting of large numbers of individual cells, each surrounded by a thick cell wall. They grow faster (minimum doubling time of 1.5 d) but have a poor affinity for acetate with K_s of 400 $mg.L^{-1}$ at pH 7. In contrast, *Methanosaeta sp.* is a filamentous organism, growing slowly with minimum doubling times of 4 d under mesophilic conditions. Their survival is due to their high affinity for acetate (*Ks* of 20 $mg.L^{-1}$ at pH 7). Consequently, *Methanosaeta sp.* will be the dominant acetoclastic species at low substrate concentration whereas high acetate concentrations favour *Methanosarcina* due to its faster growth. Also, with increasing substrate concentrations, *Methanosarcina sp.* would tend to dominate progressively more.

ii. Hydrogen-utilising Methanogenic Bacteria

Up to 30 % of the total methane production within anaerobic digesters is produced by the hydrogen-utilising methanogenic bacteria. These methanogens reduce carbon dioxide, formate, methanol and methylamines, produced earlier in the digestion process, utilising hydrogen. As a result, the methanogens grow as chemolithotrophic autotrophs because they derive both their energy and cellular carbon from inorganic chemicals.

However, such energy metabolism does not involve conventional cytochromes for electron transport. Instead, a complex seven step process has evolved with specific cofactors, such as coenzyme M (CoM), which is unique to the methanogens. CoM is the smallest coenzymes known and exceptional in its high sulphur content and acidity. The other implication of using only carbon dioxide, or other one carbon (C_1) substrates for growth is the need to generate two-carbon (C_2) building blocks for growth. Methanogens achieve this in a manner similar to that of the homoacetogenic bacteria (Zeikus et al., 1985).

2.2.2 Operational Factors Affecting Anaerobic Digestion

As discussed above, effective anaerobic degradation of organic matter requires not only healthy populations of the relevant bacterial groups but also suitable environmental conditions to support microorganism activities. Furthermore, relevant process design is directed to maintain a large and stable population of methanogens in an anaerobic digester. As a result of the critical nature of the syntrophic relationships within anaerobic treatment processes, environmental conditions require stringent monitoring and control if process failure is to be

avoided. Consequently, the environmental and operational factors known to influence digestion performance are:

i. Environmental factors: these are pH, alkalinity, temperature, nutrient availability and concentration of potential toxic compounds

ii. Operational factors: these are Solids Retention Time (SRT), Hydraulic Retention Time (HRT), Organic Loading Rate (OLR) and substrate characteristics (composition, biodegradability and concentration).

2.2.2.1 pH and Alkalinity

Anaerobic bacteria, particularly methanogens exhibit a characteristic sensitivity to extremes of pH. Therefore, a suitable and stable pH should be maintained to ensure efficient methanogenic digestion. The methanogens have a specific pH range for optimal growth, with the desired pH for anaerobic treatment being between 6.6 and 7.6 (Rittmann and McCarty, 2001). Values outside this range can be quite detrimental to the process, particularly the methanogenesis step. This is due to the fact that the hydrogen ion concentration has a critical influence on the microorganisms responsible for anaerobic digestion, the biochemistry of digestion, alkalinity buffering and several other chemical reactions affecting the solubility and availability of dissolved ions.

There are four types of chemical and biochemical reactions that influence the pH of a digester, according to Anderson and Yang (1992):

i. Ammonia consumption and release.

ii. Volatile fatty acid production and consumption.

iii. Sulphide release by dissimilatory reduction of sulphate or sulphite.

iv. Conversion of neutral carbonaceous organic carbon to methane and carbon dioxide.

In an effective working digester, natural processes such as bicarbonate alkalinity and the consumption of volatile fatty acids by methanogens can counter pH-reduction. However, the latter is dependent on the equilibrium between acidogens and methanogens and this can be easily upset by changes in the operational or environmental conditions (Anderson and Yang, 1992). The compounds that have a significant buffering capacity (alkalinity) in the useful region around pH 7.0 are carbonic acid, hydrogen sulphide, dihydrogen phosphate and ammonia (Anderson and Yang, 1992). When an anaerobic process is overloaded an accumulation of volatile fatty acids often occurs, resulting in a decrease in the pH of the system if sufficient buffering capacity is not available. Generally, the alkalinity needed to maintain a stable pH is largely governed by the carbonate equilibrium (Rozzi, 1994). Should the alkalinity fail to stabilise drops in pH, the recommended procedure is to stop feeding the reactor, giving the methanogens sufficient time to consume excess fatty acids and raise the pH value to an acceptable level. Another option is to increase the buffering capacity through the addition of calcium carbonate, sodium bicarbonate or sodium hydroxide. In some cases both options may be used simultaneously (Gunnerson and Stuckey, 1986).

2.2.2.2 Nutrients

The nutritional requirements of anaerobic bacteria are of paramount importance. This is because nutrients supply the basic cellular building blocks for growth and

ensure the cell is able to synthesise the enzymes and cofactors that drive the biochemical and metabolic reactions. Sufficient nutrient supply will enable prolonged microbial growth and conversion rates. Usually, the anaerobic process requires lower amounts of N and P than aerobic processes, due to lower biomass yields, and macronutrient (N : P) addition can be reduced up to 5 times, compared to aerobic treatment (Owen, 1982). Nutrients can be divided into two groups, the macronutrients and micronutrients. It is essential for both types of nutrients to be present in an available form in the growth environment to allow effective uptake. Ideally, nutrient levels in an anaerobic process should be in excess of the optimum concentration required since anaerobic bacteria can be severely inhibited by even slight nutrient deficiencies. However, excessively high concentrations of nutrients can become toxic to the anaerobic bacteria (Gunnerson and Stuckey, 1986).

In addition to N and P, the sulphur (S) requirement of anaerobic bacteria should also be satisfied. This can be supplied as sulphur, sulphide, sulphite, thiosulphate, sulphate or amino acids. As for micronutrients, anaerobic bacteria require a range of trace elements for metabolism and growth such iron, cobalt, nickel, zinc, copper, manganese, molybdenum, selenium, tungsten and boron (Speece, 1996). These trace elements have been shown to be stimulatory to methanogens and restriction or lack of any one required nutrient will restrict or completely stop methanogenesis (Speece, 1996; Frostell, 1985). The overall nitrogen balance is an important consideration in anaerobic digestion. For all practical purposes, N is conserved in most anaerobic applications, although any nitrates (NO_3) present will be reduced to nitrogen gas and exit as biogas. Since biomass yields are very

low, only a small fraction of the biodegradable nitrogen compounds will be converted to biomass. Most of the biodegradable nitrogen is converted to ammonia in aqueous solution. Accordingly, ammonia concentration in the effluent of anaerobic reactors is generally higher than the influent concentration (Owen, 1982).

2.2.2.3 Toxicity and Inhibition

Methanogens are commonly considered to be the most sensitive to toxicity of all the microorganisms in anaerobic degradation (Speece, 1983). According to Speece (1996), the meaning of toxicity is an adverse effect on bacterial metabolism (not necessarily lethal), while inhibition is an impairment of bacterial function. Some of the common toxic effects in waste and wastewater treatment are given below:

i. Ammonia

Ammonia is released by the fermentation of amino acids and proteins and the breakdown of methylamine and other nitrogenous compounds (Anderson and Yang, 1992). Although ammonia is an important buffer in the anaerobic digestion process and an essential nutrient for microorganisms, high concentrations can lead to operational failure. Free ammonia has been found to be much more toxic than the ammonium ion, and thus ammonia toxicity thresholds are very sensitive to pH below 7.0. In general, free ammonia levels should be kept below 80 $mg.L^{-1}$, to prevent inhibition (Anderson *et al.*, 1982). In unadapted cultures, a free ammonia level of 150 $mg.L^{-1}$ can cause growth inhibition, especially of acetoclastic methanogens (Braun *et al.*, 1981). A

combination of thermophilic conditions and high ammonia concentration has been shown to give an inhibitory effect at an ammonia concentration of 850 $mg.L^{-1}$ (Angelidaki and Ahring, 1994). The maximum concentration of free (dissolved) ammonia should not exceed the inhibitory threshold of 150 $mg.L^{-1}$ quoted by Kasapgil, (1994), although depending on the operating pH, reactors may be acclimatised reliably to NH_3-N concentrations of several thousand mg.L.

ii. Sulphide

Inorganic forms of sulphur present in reactor feeds, mainly as sulphate, are rapidly converted by sulphate reducing bacteria (SRB) to the reduced form, sulphide (S^{2-}), and hydrogen sulphide (H_2S) which are ranked as important inhibitors of anaerobic digestion (Anderson *et al.*, 1982). Sulphides in anaerobic digesters can also result from the presence of other sulphur containing compounds in the feed, and will be prevalent during anaerobic degradation of proteins (McCarty, 1964). SRB utilise the carbon source provided by the hydrolytic and acidogenic bacteria, because they are unable to produce the hydrolytic enzymes necessary for protein, carbohydrate and lipid hydrolysis (Watson and Pletschke, 2006).

According to Vela *et al.* (2002), the main problems associated with the presence of high sulphate concentrations in the influent of anaerobic reactors are recognised as:

i. Competition between sulphate reducing bacteria (SRB) and methane-producing archaea (MPA) for the same substrates (H_2, acetate).

ii. Sensitivity of MPA to sulphide, leading to methanogenesis inhibition when the sulphide concentration exceeds certain limits.

iii. Precipitation of trace metals, causing nutritional deficiencies in the reactor.

The result of competition between two different groups of bacteria, the methane producing bacteria (MPB) and the SRB will define the composition of the biogas and thus the feasibility of treatment by a methanogenic process (Freese and Stuckey, 2004). MPB and SRB competition studies have found that neither group is consistently dominant, and it has been shown that under the same conditions either bacterial group can dominate (O'Flaherty et al., 1998). SRB are more likely to be involved in the following processes of anaerobic digestion:

i. Competition between the SRB and homoacetogenic bacteria for hydrogen, and

ii. Competition between the SRB and methanogens for direct methanogenic precursors such as acetate and H_2 (O'Flaherty et al., 1998).

According to Chynoweth *et al.* (1999), up to 150 $mg.L^{-1}$ H_2S does not cause microbial inhibition, but can be problematic when present due to its odour and potential human toxicity. Maillacheruvu and Parkin (1996) showed that the H_2S toxicity of the hydrogen-utilising methanogens is relatively weaker than for other microbial groups, explaining how methanogenesis can occur from complex substrates even at high concentration of sulphide. In sulphide toxicity control, it is important to diagnose the inhibition first, and then take the necessary actions (Anderson et al., 2003). This requires close process monitoring in either the

liquid / slurry phase or the gas phase. The former should include measurements of pH, total and individual VFA, alkalinity, COD, BOD and solids. The latter usually involves measurement of gas production rate and gas composition (methane and carbon dioxide). In addition to these chemical analyses, microbiological analyses (enumeration of anaerobic bacteria by microscopy, agar plate count and MPN), and biochemical analyses (ATP, co-enzymes F_{420}, specific methanogenic activity (SMA) and dehydrogenic activity) have been undertaken to give an early indication of metabolic inhibition (Kasapgil, 1994). Speece (1996) has recommended the following strategies to control sulphide toxicity in anaerobic processes:

i. Raising the pH so that H_2S is converted to the less toxic HS^- form,
ii. Chemical scrubbing (e.g. iron sponge soaked with $FeCl_3$) and recycling the reactor gas,
iii. Precipitating the sulphide with iron salts,
iv. Inhibiting the SRB with molybdate,
v. Implementing two-phase operation,
vi. Using thermophilic conditions.

iii. Volatile Fatty Acids

High concentrations of VFA are often associated with the effects of toxicity and inhibition. It is well documented that high VFA concentrations in the anaerobic processes cause the inhibition of methanogenesis (Marchaim and Krause, 1993). Generally, VFA inhibition occurs due to their accumulation and a consequent reduction in pH value. This is due to the fact that under conditions of overloading and in the presence of inhibitors, methanogenic activity cannot

remove hydrogen and volatile organic acids as quickly as they are produced. As a result, the accumulation of acids and the depression of pH reach a level that also inhibits the hydrolysis or acidogenesis phase. It has also been shown that even when process pH is optimal, the accumulation of VFA may contribute to a reduced rate of hydrolysis of the solid organic substrate (Banks and Wang, 1999). Inhibition of the fermentative bacterial population by its main product VFA when using glucose as the main substrate has also been observed (van den Heuvel *et al.*, 1992). More recently, Siegert and Banks (2005) showed that VFA caused the inhibition of the cellulolytic activity at concentrations of 2000 $mg.L^{-1}$.

iv. Metals

As discussed in Section 2.2.2.2, trace elements have been shown to be stimulatory to methanogens for the function of certain enzymes and co-enzymes, however excessive amounts may result in toxicity or inhibition. Heavy metal toxicity is believed to occur through the structural disruption of enzymes and protein molecules within the cell (Hickey et al., 1989). Several effects of metal ion toxicity have been observed. For example, Mehrotra *et al.* (1987), revealed that the relative toxicity of zinc (Zn), lead (Pb) and chromium (Cr) appeared to decrease in the order Zn > Pb > Cr, while Hickey *et al.* (1989), reported that the relative toxicity Copper (Cu), Cadmium (Cd) and Zn was Cu > Cd > Zn. Fang (1997) revealed that the relative toxicity of zinc (Zn), nickel (Ni), copper (Co), cadmium (Cd) and chromium (Cr) appeared to decrease in the order Zn > Ni > Co > Cd > Cr in an UASB reactor treating synthetic wastewater.

v. ***Oxygen***

Methanogenic bacteria are very sensitive to oxygen exposure. Therefore, highly reduced environments (absence of oxygen) should be maintained to promote obligate anaerobic bacteria (Pfeffer, 1979). However, there are also likely to be facultative anaerobic bacteria present in an anaerobic digester. Therefore, any oxygen present will be rapidly consumed by these microorganisms allowing an effective production of methane. Furthermore, anaerobic bacteria often exist in structured communities (e.g. granules, biofilms and flocs) where the outer layers of cells are responsible for creating an anoxic or anaerobic core micro-environment suitable for the sensitive methanogens.

2.2.2.4 Temperature

Temperature is one of the most influential factors in anaerobic digestion since the rate of biochemical and enzymatic reactions within cells will generally be increased by increasing temperatures causing increased growth rates. Rittmann and McCarty (2001) stated that the growth rate of microorganisms doubles for each 10°C rise in temperature within the mesophilic operational range. Although methane formation is biologically feasible at all temperatures between 0°C and 100°C, treatment of wastewater in anaerobic reactors is normally carried out within two different temperature ranges. These are known as the mesophilic range (25 - 40°C) and the thermophilic range (>45 °C) (van Lier *et al.*, 1996). A third range, favoured by psychrophilic organisms has an optimum temperature of 15 - 20°C.

Although not as efficient as high rate mesophilic and thermophilic digestion, psychrophilic digestion may still have desirable economic trade-offs for the anaerobic treatment of wastewater in temperate climates. It is generally believed that thermophilic reactors are more efficient than mesophilic reactors and bench-scale experiments reveal methane production rates in thermophilic reactors can be double that of mesophilic reactors. A thermophilic reactor can also accept higher organic loading rates and produce lower sludge quantities. Dugba and Zhang (1999) reported that physical parameters such as viscosity and surface tension will change with temperature, and improved mass transfer, and higher degradation rates have been shown under thermophilic conditions. However, Fang and Wai-Chung Chung (1999), reported that a number of disadvantages have been observed for full-scale thermophilic digesters such as low cell yield, lower stability than mesophilic and reduced degradation rate. Nevertheless, thermophilic anaerobic digestion is an attractive option for treating warm industrial effluents and slurries of relatively constant composition (Lettinga, 1995). Compared to many aerobic processes that are relatively robust to temperature variations, anaerobic digestion is particularly sensitive to sudden temperature fluctuations, changes as small as 1 – 2°C having significant adverse effects on process performance especially when changes occur rapidly (less than 2 hours) (Uyanik, 2001). Should the bacteria become adversely affected by digester temperature variations, several days or even weeks may be required to restore a healthy population once again.

Furthermore, it has been concluded that in anaerobic baffled reactors (ABR), COD in the effluent increases as the temperature decreases (Nachaiyasit, 1995)

indicating the effect of temperature on process performance. In addition, production of soluble microbial products (SMP) in ABRs increases with decreasing temperature, probably due to increased stress on the biomass and a reduced metabolism of the SMP at low temperature (Barber et al., 2000).

2.2.2.5 Mixing

Mixing is an important process as it helps to improve the contact between organic matter and the microorganisms for higher reactor performance. However, methanogenic anaerobic digesters have an inherent degree of mixing from the continuous stream of methane bubbles that rise within the reactor. This natural mixing is usually considered to be rate limiting for efficient mass transfer. The level and type of mixing also affects the growth rate and distribution of microorganisms within the sludge, substrate availability and utilization rates, granule formation and gas production (Smith *et al.*, 1996).

In anaerobic process, mixing is accomplished by one or a combination of the three usual methods; liquid recirculation by pump, gas produced is compressed and injected into the liquid and mechanical mixing (Rittmann and McCarty, 2001). Stafford (1981) reported that as long as adequate mixing was achieved, the method of mixing had little bearing on the digestion rate. However, it was also reported that excessive mixing could actually lead to a reduction in reactor performance. McMahon *et al.* (2001) found that continuously mixing reactors could lead to inhibition of the syntrophic oxidation of volatile fatty acids, possibly by disrupting the spatial juxtaposition of syntrophic bacteria and their methanogenic partners. Subsequently, no matter how the mixing is effected, the

aim is to maintain high enough liquid velocity so that all the solids in digester remain in suspension.

2.3 SULPHATE-REDUCTION PROCESS

The production of hydrogen sulphide is a well known manifestation of anaerobic processes. According to Gray (2004), sulphide can be produced by anaerobic microorganisms in two ways. Protein is broken down to amino acids and those containing sulphur (e.g. cysteine and methionine) are degraded further with sulphide being produced. This can be done by most anaerobic bacteria such as *Proteus*, *Bacteroides* spp., and some *Clostridium* spp. Furthermore, in wastewater systems, most sulphide is produced from sulphate reduction by the anaerobic sulphate-reducing bacterium *Desulfovibrio desulfuricans*, although species of the genus *Desulfotomaculum* are routinely isolated from digesters (Zeikus, 1980). The sulphate-reducing bacteria only utilise a restricted range of carbon compounds, such as lactate and malate and rely on the metabolic products of other anaerobic bacteria that are able to utilise more complex organic compounds (Gray, 2004). A study by Greben et al., (2000) found that methanol induced methanogenesis rather than sulphidogenesis and further concluded that sugar and ethanol were found to be suitable carbon and energy sources for sulphate reduction. The reaction for SRB in sulphate reduction is as follows:

$$8e^- + SO_4^{2-} + 8H^+ \longrightarrow S^{2-} + 4H_2O$$

From the above equation, oxidation of 64 gram COD is required for the reduction of 96 gram SO_4^{2-} to 32 gram S^{2-}.

2.3.1 Microbiology of Sulphate-Reducing Bacteria

Desulfovibrio is the dominant genus in sulphate-reducing bacteria which uses sulphate reduction to sulphide as the main terminal step in the anaerobic oxidation of a limited range of organic substrates such as the oxidation of lactate to acetate (Postgate, 1984). Apart from *Desulfovibrio*, there are many other genera which differ in morphology and physiology and can be divided into three groups, namely nonacetate oxidizers, acetate oxidizers and dissimilative sulphur reducers. The range of electron donors used by these sulfate-reducers is fairly broad e.g. hydrogen, lactate, pyruvate, malate etc.

Acetate oxidizing bacteria (*Desulfobacter, Desulfobacterium, Desulfococcusdiffer* etc.) differ from nonacetate oxidizers (*Desulfovibrio, Desulfomicrobium*, etc.) by their ability to oxidize fatty acids (including acetate), lactate and succinate completely to CO_2. *Desulfosarcina, Desulfonema, Desulfobacterium, Desulfotomaculum* and certain species of *Desulfovibrio* are unique among sulphate-reducers in their ability to grow chemolithotrophically and autotrophically with H_2 as electron donor, sulphate as electron acceptor and CO_2 as the sole carbon source (Madigan and Martinko, 2006).

2.3.2 Operational Factors Affecting the Sulphate-Reduction Process

As for other anaerobic processes, the effectiveness of the sulphate-reduction process requires suitable environmental conditions to support sulphate-reducing activities. In natural habitats, the capacities to adapt to modifications of biological and physico-chemical factors may be decisive for the growth and

activity of a microorganism (Barton, 1995). In this section, the effect of pH, temperature and substrate on sulphate-reducers will be briefly discussed.

2.3.2.1 pH

Sulfate-reducing bacteria grow better under slightly alkaline conditions over a relatively restricted pH range of 7.0 to 7.8 but tolerate pH values ranging from 5.5 to 9.0 (Pfenning et al., 1981). Zehnder (1988) reported that sulphate-reducing bacteria were usually inhibited at pH values lower than 6 or higher than 9. However, they can compensate for a high pH value if long-chain fatty acids serve as electron donors. A study by O'Flaherty et al., (1998) reported that between pH 7.0 and 7.5 the growth rates of the SRB and MPB are similar, however, SRB have better growth properties than MPB at pH above 7.5. Another study by Elliot et al., (1998) on upflow anaerobic bioreactor reported that SRB were capable of sulphate reduction at pH values as low as 3.25.

2.3.2.2 Temperature

Mesophilic sulphate reducers grow best between 28 and 38°C and have an upper temperature limit around 45°C (Widdel and Hansen, 1992). The optimum growth temperature for thermophilic eubacterial sulphate reducers of the genera *Desulfotomaculum* and *Thermodesulfobacterium* ranges from 54 to 70°C (Zehnder, 1988).

2.3.2.3 Substrate

Substrate plays a vital part in the competition of SRB in anaerobic system. However, in anaerobic environments SRB cannot effectively compete against

the fast-growing fermentative bacteria involved in polymer hydrolysis and monomer degradation, so they are more likely to be involved in the competition with homoacetogenic bacteria for hydrogen and with methanogens for acetate and H_2 (Freese and Stuckey, 2004). The COD: sulphate ratio is usually used as a qualitative reference to determine when SRB should begin to outcompete the MPB. The major change is at a COD: sulphate ratio of 1 - 2 (Freese and Stuckey, 2004).

A study by Freese and Stuckey, (2004) using an anaerobic baffled reactor (ABR) showed that at a COD: sulphate ratio of 1, total COD removal was around 77 %, with around half of the COD removal (38.5 %) being due to the destruction of sulphate throughout the reactor. They suggested possible reasons why COD removal via methanogenesis decreased, including the precipitation of Fe by sulphide ions which hampered methane production, the faster growth rates of the sulphidogens and direct H_2S inhibition of the MPB. If values are less than 1 then SRB are likely to predominate; if greater than 2, then the MPB will dominate (Speece, 1996; O'Flaherty et al., 1998). Furthermore, a COD:sulphate ratio greater than 10 should lead to insignificant sulphide inhibition, posing no problems for methanogenesis (Rinzema and Letingga, 1988).

2.3.3 Sulphate Reduction during Anaerobic Digestion

Hydrogen sulphide production is most common and well-known in anaerobic processes. Sulphate-reducing bacteria are found in a wide range of anaerobic environments where there is a supply of sulphate, which they utilise instead of oxygen for respiration, organic matter and a suitable bacterial population able to

utilise the complex organic matter to produce simpler compounds such as lactate (Lynch and Poole, 1979). There are three general relationships between sulphate-reducing bacteria and methane-producing bacteria proposed by Odom and Singleton in 1993:

i. competition between two groups for limiting electron donor (organic acids, acetate and hydrogen);
ii. coexistence through use of separate resources;
iii. a synergism in which members of one of the two groups supply an electron donor needed by the other.

Competition. Acetate and hydrogen are two electron donor used by both groups which sulphate-reducing bacteria has higher affinity over methane-producing bacteria provided they have sufficient supply of an electron acceptor which is sulphate (SO_4^{2-}). If the SO_4^{2-} concentration is abundant, sulphate reducers are dominant in the competition and vice versa.

Coexistance. These two groups of bacteria can utilise different electron donors when present in the same microenvironments. Oremland and Policin (1982) reported that acetate and H_2 can both be utilized by sulphate-reducing bacteria and methane-reducing bacteria but further observation showed that sulphate-reducing bacteria could not use methanol, trimethylamine and methionine as electron donors.

Synergism. Bryant et al., (1977) suggested that some sulphate-reducing bacteria obtain small amount of energy from the fermentation of lactate to acetate and H_2

which cannot be metabolized by methane-producing bacteria. This transformation has made it possible for methane-producing bacteria to utilise acetate and H_2 and showed close metabolic association between sulphate-reducing bacteria and methane-producing bacteria.

Table 2.1: Studies on the competition between Sulfate Reducing and Methanogenic bacteria in anaerobic process.

References	System	Retention Time (h)	C source
Freese and Stuckey, 2004	Anaerobic baffled reactor (ABR)	20-80	Sucrose
Mizuno et al., 1994	Anaerobic chemostat type reactor	120-480	Butyric acid
Weijma et al., 1999	EGSB	3.5-14	Methanol
Omil et al., 1998	UASB	6	VFA mixture
Raskin et al., 1996	Fix-bed biofilm reactor	-	Glucose

EGSB=expanded granular sludge bed reactor

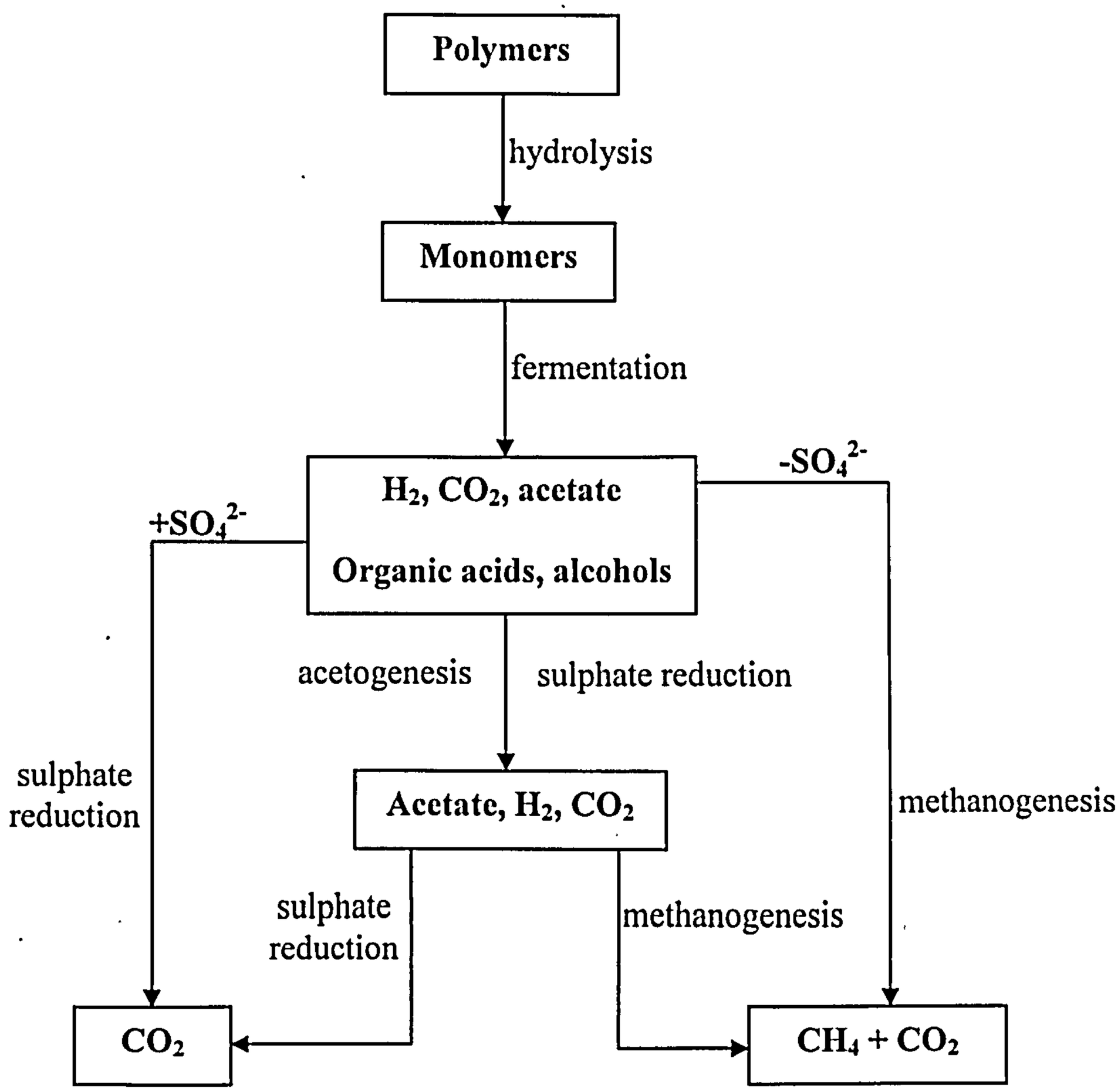


Figure 2.3: Potential competition of SRB for methanogenic substrates during the degradation of organic matter in anaerobic digester (adapted from Barton, 1995).

2.4 DENITRIFICATION

Although anaerobic treatment has been widely applied in many sectors, denitrification is still needed to eliminate nitrogenous compounds in wastewater to concentrations below those specified in the legislation. This can be done in a single unit; where anaerobic digestion and denitrification take place simultaneously, producing methane and nitrogen gas. Denitrification refers to the process of converting nitrate via nitrite to gaseous nitrogen (N_2) under low

dissolved oxygen conditions and these bacteria degrade COD using nitrite ions and nitrate as the primary electron acceptors. Denitrification occurs in the following sequence:

$$NO_3 \xrightarrow[\text{reductase}]{\text{Nitrate}} NO_2 \xrightarrow[\text{reductase}]{\text{Nitrite}} NO \xrightarrow[\text{reductase}]{\text{Nitrate oxide}} N_2O \xrightarrow[\text{reductase}]{\text{Nitrous oxide}} N_2$$

2.4.1 Microbilogy of Denitrifying Bacteria

Denitrification can be carried out by several groups of organisms including fungi and the protozoa *Loxodes*. Most of these denitrifying organisms consists of facultative anaerobic bacteria and are known by several names such as denitrifiers, heterotrophs and organotrophs. Denitrifying bacteria degrade COD using nitrite ions and nitrate ions in the absence of free molecular oxygen. The bacteria degrade COD in order to obtain energy for cellular activity and carbon for cellular synthesis (growth and reproduction).

Inorganic nitrogen compounds such as nitrate, nitrogen dioxide, nitrite and nitrogen oxide are the most common electron acceptors in anaerobic respiration (Madigan and Martinko, 2006). These nitrogen containing ions occur widely in a variety of process streams, such as those coming from extensive use of fertilizers (Beschkov *et al.*, 2004). A relatively large number of genera of facultative anaerobes are capable of denitrification. Most denitrifiers reduce nitrate via nitrite to molecular nitrogen without the accumulation of intermediates.

However, some denitrifiers lack key enzyme systems to denitrify completely and the lack of these enzyme systems does permit the production and accumulation of intermediates (Gerardi, 2002). Although there are numerous genera of denitrifying bacteria, these denitrifying genera do not contain a large number of species and all denitrifying bacteria do not respire similarly. The genera *Alcaligenes*, *Bacillus* and *Pseudomonas* contain the largest number of denitrifying bacteria while *Pseudomonas, Achromobacter* and *Bacillus* are some of the bacteria with high denitrifying capabilities (Payne, 1981). Most genera of denitrifying bacteria can use either nitrite or nitrate to degrade COD, however, some genera such as *Enterobacter* and *Escherichia* can use only nitrite (Gerardi, 2002). The use of nitrate in this manner is known as nitrate respiration, while the use of nitrite is known as nitrite respiration (Gerardi, 2002).

The reduction of nitrate to only nitrite during denitrification may result in an accumulation of nitrite. Some genera of denitrifying bacteria are microaerophilic but can tolerate only low levels of free molecular oxygen. Some genera of denitrifying bacteria including species of *Corynebacterium* and *Pseudomonas* do not denitrify completely and produce nitrous oxide instead of molecular nitrogen as their gaseous end product (Gerardi, 2002). Most denitrifying bacteria cannot ferment, that is, use one molecule of COD to degrade another molecule of COD. However, some species of *Bacillus* and *Chromobacterium* can denitrify and ferment at the same time (Gerardi, 2002). Finally, species of *Propionicbacterium* that denitrify cannot respire aerobically, that is, cannot use free molecular oxygen. The enzymatic machinery needed for denitrification is formed only under anoxic conditions with the presence of a low oxygen concentration

(Gerardi, 2002). However, the production of the enzymatic machinery for denitrification is accomplished quickly when conditions change (Gerardi, 2002).

2.4.2 Operational Factors Affecting Denitrification

Operational factors play an important role as they strongly influence denitrification, these include substrate type, the absence of free molecular oxygen, the presence of an adequate and active population of denitrifying bacteria, pH, temperature, nutrients, and redox potential.

2.4.2.1 Substrate

The quantity of substrate or COD rather than the quantity of nitrite ions or nitrate ions is considered to be most important factor that determines denitrification and this was proved by Akunna et al., (1992) in their research using laboratory-scale anaerobic digesters fed with synthetic wastewater. They found that values of COD:N ratio between 9 and 53 produce methanisation and complete denitrification while with values lower than 9, denitrification dominated. The larger the quantity of COD, especially COD as simple soluble molecules, the greater is the demand for electron acceptors, such as free molecular oxygen, nitrite ions, and nitrate ions. The greater the demand for electron acceptors, the greater are the chances that denitrification will occur (Gerardi, 2002). Denitrifying bacteria use many ordinary, organic compounds and unusual organic compounds as a source of carbon and energy, and can use organic compounds commonly found in domestic wastewater. Typical organic compounds added to a denitrification tank in a STP to fully denitrify the effluent include acetic acid, ethanol, glucose, methanol and molasses (Gerardi, 2002).

$$NO_3^- + 0.85CH_3COOH + H^+ \longrightarrow 0.1C_5H_7O_2N + 0.45N_2 + 1.2CO_2 + 2.1H_2O$$

(denitrification using acetic acid, CH_3COOH)

2.4.2.2 Absence of Free Molecular Oxygen

Free molecular oxygen inhibits denitrification by virtue of its competition with nitrite ions and nitrate ions as an electron acceptor for the degradation of COD. If free molecular oxygen is present in the environment of the bacterial cell and enters the bacterial cell, the cell uses free molecular oxygen in preference to nitrite or nitrate ions because it yields more cellular energy and cellular growth. Furthermore, excessive concentrations of molecular oxygen can lead to an accumulation of the denitrification intermediates such as nitrite, nitrogen dioxides and nitrous oxide (Rittmann and McCarty, 2001).

In relation to the enzymes involved in the denitrification process, molecular oxygen can control denitrification in two ways. The first is repression of the several nitrogen-reductase genes when the molecular oxygen concentrations are greater than 2.5 to 5 $mgO_2.L^{-1}$ (Körner and Zumft, 1989). The second control mechanism is direct inhibition of the activity of the reductase by molecular oxygen concentrations greater than a few tenths of a $mgO_2.L^{-1}$ (Tiedje, 1988; Rittmann and Langeland, 1985). The amount of oxygen that inhibits denitrification is therefore relatively small and concentrations of dissolved oxygen < 1.0 $mg.L^{-1}$ can still inhibit denitrification (Gerardi, 2002).

2.4.2.3 pH

Denitrification can occur over a wide range of pH values, and is relatively insensitive to acidity but may be slowed at low pH, the optimum pH values for denitrification being within a range of 6.5 to 8.5 (Gerardi, 2002). The pH values outside this optimal range can lead to accumulation of intermediates (Rittmann and McCarty, 2001). Gray (2004) reported that the denitrification reaction is sensitive to pH with an optimum range between pH 6.5 and 7.5, but this falls to 70 % efficiency at pH 6 or 8. In contrast, other researchers have investigated the use of control systems to lower pH, maintaining near neutral conditions and concluded that pH control did not increase the rate of denitrification, which remained the same at pH 7.5 and 9.5 (Cutter, 1992; Cook et al., 1993).

2.4.2.4 Temperature

Because denitrification is biologically mediated, denitrification occurs more rapidly with increasing temperature and conversely, denitrification occurs more slowly with decreasing temperature, being inhibited in wastewater below 5°C (Gerardi, 2002). To compensate for decreased denitrification at cold temperature, increasing the MLVSS can increase the number of denitrifying bacteria. Because denitrification is linked to nitrification and nitrification also is biologically mediated, a warmer temperature also favours rapid formation of nitrate ions. Warmer wastewater also has less affinity for dissolved oxygen than colder wastewater, and it is exhausted more readily during warm wastewater conditions, allowing denitrification to occur more efficiency. The optimum temperature for denitrification to occur is in the range of 35-50°C (Gary, 2002).

2.4.2.5 Nutrient

The major nutrient requirements for facultative anaerobes are nitrogen and phosphorus. Because of the greater energy yield and greater cell production of facultative anaerobes during aerobic respiration of COD, as compared to anoxic respiration of COD, nutrient guidelines for facultative anaerobes during aerobic respiration can be used for these bacteria during anoxic respiration. These guidelines for nitrogen and phosphorus during aerobic respiration are 1.0 $mg.L^{-1}$ for ammonium ions or 3.0 $mg.L^{-1}$ for nitrate ions and 0.5 $mg.L^{-1}$ for orthophosphate ions (HPO_4^{-2}) in the mixed liquor effluent filtrate at all times (Gerardi, 2002).

2.4.2.6 Redox Potential

Nitrite ions and nitrate ions can be used for bacterial degradation of COD at operational redox potentials of +50 to -50 milivolts (mv). Redox is the measurement of the amount of oxidized compounds, such as nitrate etc in a wastewater sample. Within the range of +50 to -50 milivolts, oxygen is either absent or present at relatively low concentration, while nitrite and nitrate can be present at relatively high concentrations.

2.4.3 Denitrification in Anaerobic Digesters

Interactions between the denitrifiers and methanogens can take place in the mixed cultures present in anaerobic digesters as shown by previous reserchers (Hanaki and Polprasert, 1989; Akunna et al., 1992). They reported in their studies using synthetic wastewaters and anaerobic upflows filters that

denitrification took place in the bottom of the digester while methanogenesis occurred in the upper part where nitrate had been completely exhausted.

Figure 2.4: Interactions between methanogenesis and denitrification in an Anaerobic Digester (adapted from Fang and Zhou, 1999).

Methanogens will compete with denitrifiers for carbon substrates· but denitrification is more competitive for those substrates when nitrate or nitrite is present. Methanogenic activities only begin after denitrification is complete and the excess organic carbon remaining can then be utilized by methanogens to produce methane and carbon dioxide.

Furthermore, nitrate has been found to strongly inhibit methanogenesis (Akunna et al., 1993; Allison & Macfarlane, 1988; Scholten & Stams, 1995; Hendriksen & Ahring, 1996; Chen & Lin, 1993; Lin & Chen, 1995). This inhibitory effect is still unclear and cannot be attributable only to a redox change (Britz & van der Merwe, 1993; Sorensen, 1978; Allison & Macfarlane, 1988; Westermann & Ahring, 1987). Chen and Lim (1992) proposed that the inhibitory effects caused by nitrate and nitrite on methane production may be due to their toxic effect on enzyme systems and/or changes in the redox potential.

In contrast Clarens et al., (1998) suggest that the growth of denitrifying organism was responsible for methane production failure rather than nitrate itself. This observation was supported by Akunna et al., (1992) who found no inhibitory effect on methanogenic was observed up to concentrations of 800 mg NO_3^--Nl^{-1}. Nitrate and nitrite transferred to an anaerobic digester will have two significant and adverse impacts on digester performance.

First, the rapid depletion of these ions through denitrification in the digester and the release of molecular nitrogen results in sudden and severe foaming; and second, their presence increases the redox potential of the digester sludge

(Gerardi, 2002). A study by Klüber and Conrad (1998) on the inhibitory effects of nitrate and its denitrification intermediates on methanogenesis concluded that nitrite was a more effective inhibitor than nitrate with only micromolar concentrations were being sufficient to completely inhibit methanogens.

Table 2.2: Denitrification with methanogenic sludge (adapted from Mosquera-Corral et al., 2001).

References	System	C source	HRT (h)	NO_3^--N removal ($g\ Nl^{-1}d^{-1}$)
Jorgensen and Tiedje (1993)	Batch	Several	-	0.087
Quevedo et al. (1996)	Batch	Glucose Acetic acid EAIR[c]	- - -	0.583[a] 1.648[a] 0.768[a]
Akunna et al. (1992)	CSTR	Glucose	10	0.2
Rustrian et al. (1997)	CSTR	Acidogenic	0.52	0.684
Hanaki and Polpraset (1989)	AF	Methanol	0.16	1.3
Battistoni and Fava (1995)	Activated sludge	RDCOD[b]	1	0.2[a]
Mosquera-Corral et al (2001)	USBF	EAIR[c]	18	-

[a] g N g VSS^{-1} D^{-1}
[b] RDCOD=readily degradable COD
[c] EAIR=effluent from anaerobic industrial reactor

2.5 ANAEROBIC MEMBRANE BIOREACTOR (AMBr)

The AMBr can be considered as integrating two separate systems, a bioreactor for biological activity and a membrane filter for solids retention. Membrane filtration has received much interest recently as a means of biomass retention in wastewater treatment systems (Fuchs et.al., 2003).

The membrane unit can be configured externally to the reactor, as a sidestream operation, or submerged internally within the bioreactor (Figure 2.5). In the external system (sidestream operation) the membrane unit operates separately

from the reactor. Wastewater influent enters the bioreactor where it contacts the micro-organisms of the mixed liquor. The mixed liquor is then pumped continuously around a recirculation loop containing a membrane filtration unit which generates a retentate flow and a permeate flow. The latter provides the reactor effluent whilst the former is returned back to the bioreactor. The recirculation is driven by a pump that generates sufficient crossflow velocity and transmembrane pressure (TMP) to provide adequate permeate flux and reduce gel-layer formation.

Being the first to be developed, the sidestream system has been used in industrial wastewater applications, drinking and groundwater treatment for several decades, and has generally been considered to be more suitable for wastewater streams characterized by high temperature, high organic strength, extreme pH, high toxicity and low filterability (Yang et al., 2006).

The sidestream system operates at a higher TMP, and therefore has a higher flux than submerged systems, which operate at lower Reynolds number. High circulating flux also causes excellent mixing conditions inside the bioreactor between influent and biomass. For example, efficient mixing conditions have been reported for a MBR treating maize processing effluent at a recycle ratio of 1:6, however, this required a power consumption, which was twice the recommended value of the US EPA (Ross et al., 1992). The main advantage of the sidestream system is the improved control of concentration polarisation of the membrane which reduces membrane fouling and a lower surface area requirement compared to submerged membrane systems.

In the submerged membrane system there is no recirculation loop because the membrane unit is immersed directly within the bioreactor. In this configuration, the TMP is driven by the hydrostatic head generated by the liquid level above the membrane, although this can be increased by using a suction pump on the permeate line. Control of membrane fouling can be achieved by continuously scouring the membrane surface with a recirculation of biogas. Pioneering work by Hu & Stuckey (2004) has shown that the submerged anaerobic membrane bioreactor (SAMBR) treating dilute wastewater can be operated for extended periods without excessive membrane fouling and has a comparable efficiency with most other forms of anaerobic treatment.

However, both systems retain almost all of the biomass within the bioreactor leading to excellent effluent quality capable of meeting stringent discharge requirements and provides opportunities for direct water reusė (Chiemchaisri et al., 1992). The ability to retain almost all bacteria results in a near sterile effluent, eliminating the need for extensive disinfection and the corresponding hazards related to disinfection by-product formation (Yang, et al., 2006).

Furthermore, it provides an efficient tool for maintaining a long solid retention time (SRT) at a relatively short hydraulic retention time (HRT). While a high SRT is preferred for process stability, a short HRT minimizes the reactor volume and hence, reduces capital costs (Hütter et al., 2000).

In contrast, the conventional anaerobic digester is a completely mixed reactor with no solids recycle, in which the solids retention time equals the hydraulic

retention time (Anderson et al., 1986). Advantages of the AMBr include small footprint, complete solids removal from effluent, effluent disinfection, high loading rate capability, low or zero sludge production, rapid start-up, efficient removal of COD, solid and nutrient in a single unit and freedom from the effects of sludge bulking. Because of these advantages, AMBr are becoming increasingly popular in the field of environmental engineering worldwide (Table 2.3). Although the AMBr has been widely used in wastewater treatment systems, several promising areas of AMBr application remain unexplored and require detailed experimental evaluation. These include treatment of wastes generated from agricultural sources and livestock operations, wastewater originating from food processing industries, removal of herbicides, pesticides and endocrine disrupting substances from wastewater and water streams (Fonseca et al., 2000; Mansell and Schroeder 1998; Nah et al., 2000; Urbain, 1996).

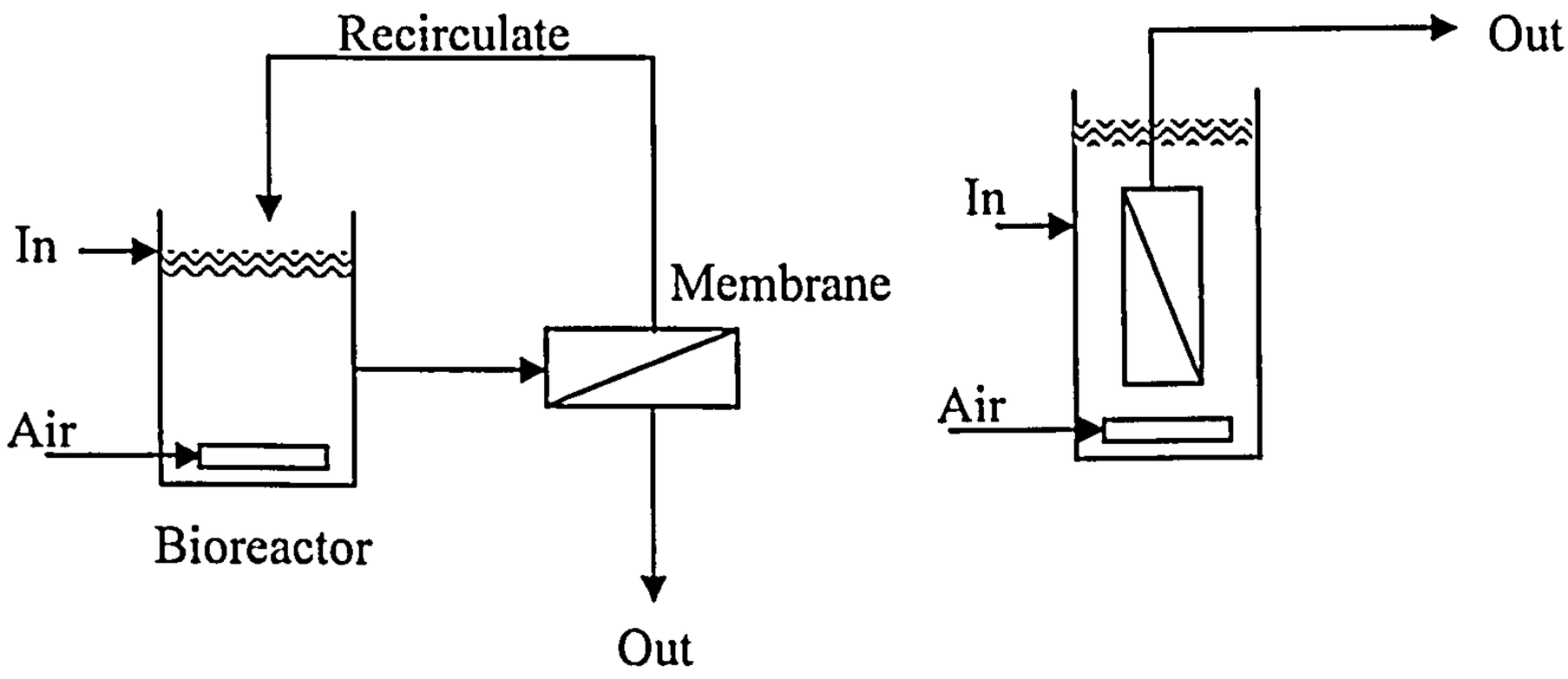


Figure 2.5: Configurations of MBRs: Sidestream (left) and submerged (right)(taken from Stephenson et al., 2000).

2.5.1 Loading rates

Laboratory scale research carried out by Anderson et al., (1986) reported that a maximum loading rate of 54.1 $kgCOD.m^{-3}.d^{-1}$ for acidogenic bacteria and 12.2 $kgCOD.m^{-3}.d^{-1}$ for methanogenic bacteria with an average 99.04% COD reduction obtainable in methanogenic reactor. Fuchs et al., (2003) in their laboratory plant studies treating wastewater with high organic content found that more than 90% COD removal efficiency was obtainable up to a maximum loading rate of 20 $kgCOD.m^{-3}.d^{-1}$.

Removal efficiencies of more than 90% for a range of wastewaters treated with an AMBr have also been reported elsewhere (Li et al., 1985; Kayawake et al., 1991; Strohwald and Ross, 1992; Ince et al., 1998; Fakhru'l-Razi and Noor, 1999). The AMBr has also demonstrated stability for COD removal over a wide range of loading rates, and a scale study using synthetic wastewater, found that a drop of just 7% in COD removal efficiency occurred as the organic loading rate was increased from 7.7 to 24.2 $kgCOD.m^{-3}.d^{-1}$ (Cadi et al., 1994).

2.5.2 Gas Production

The production of methane typically provides benefits of about 22 to 26 $MJ.m^{-3}$ energy recovery, depending on the carbon dioxide content of the biogas (Stephenson et al., 2000). Reported methane yields of AMBr range between 0.17 to 0.29 $m^3CH_4.kgCOD^{-1}$ destroyed but the actual yield is heavily dependent on the source of wastewater and operating conditions (Strohwald and Ross, 1992; Hogetsu et al., 1992; Fakhru'l-Razi and Noor, 1999; Ince et al., 1998). The theoretical methane yield is 0.35 $m^3CH_4.kgCOD^{-1}$ destroyed (Li et al., 1985) with proportions of 65 to 75% in the biogas being considered indicative of good

performance (Strohwald and Ross, 1992). Several factors will affect the amount of biogas production such as organic loading rate, HRT and temperature. Although gas production usually increases up to a point with increasing OLR, increasing loading rate excessively (Ince et al., 1998; Fakhru'l-Razi and Noor, 1999; Fuchs et al., 2003) and HRT (Cadi et al., 1994) can have an adverse effect on biogas production. Hogetsu et al., (1992) found that biogas production was increased when operation was switched from mesophilic (37°C) to thermophilic (53°C) digestion.

2.5.3 Biomass

There have been several studies on the characteristics of biomass anaerobic membrane bioreactors (Kataoka et al., 1992; Harada et al., 1994; Ince et al., 1998). Kataoka et al., (1992) observed that bacterial populations treating sewage grew slower than during the treatment of some industrial wastes. This phenomenon has been explained by the large amounts of cellulosic materials that are generally present in sewage, hydrolysis of which is known to be the rate limiting step (Klass, 1984). Ince et al., (1998) revealed that a shift in the dominant methanogenic group from *Methanococcus* at start up to medium-sized rods at the end of the study in an MBR treating brewery wastewater. Furthermore, they observed a sharp increase in numbers of viable organisms when resulting in a 6 fold increase in their metabolic activity.

2.6 MEMBRANE (TYPES AND PROCESSES)

Membranes can be categorized into three groups according to the particle size they retain:

i. Microfiltration (MF): 0.1 to 10 micron

ii. Ultrafiltration (UF): 0.001 to 0.1 micron

iii. Reverse Osmosis (RO): < 0.001 micron

Generally, microfiltration (MF) membranes are used for the retention of small particulates, microorganisms, viruses and colloids, whilst ultrafiltration (UF) membranes are used to recover macromolecules from solution as well as colloids. Reverse osmosis (RO) membranes are capable of rejecting ionic species such as sodium or chloride with effective exclusion sizes in the same order of magnitude as a water molecule. RO operation involves very high pressure (generally 25 to 60 atmosphere) in order to overcome the osmotic pressure which hinders the separation of salts from water. However, MF and UF operate at relatively lower pressures (typically 1 to 8 atmosphere) since macromolecules and colloids do not have significant osmotic effects.

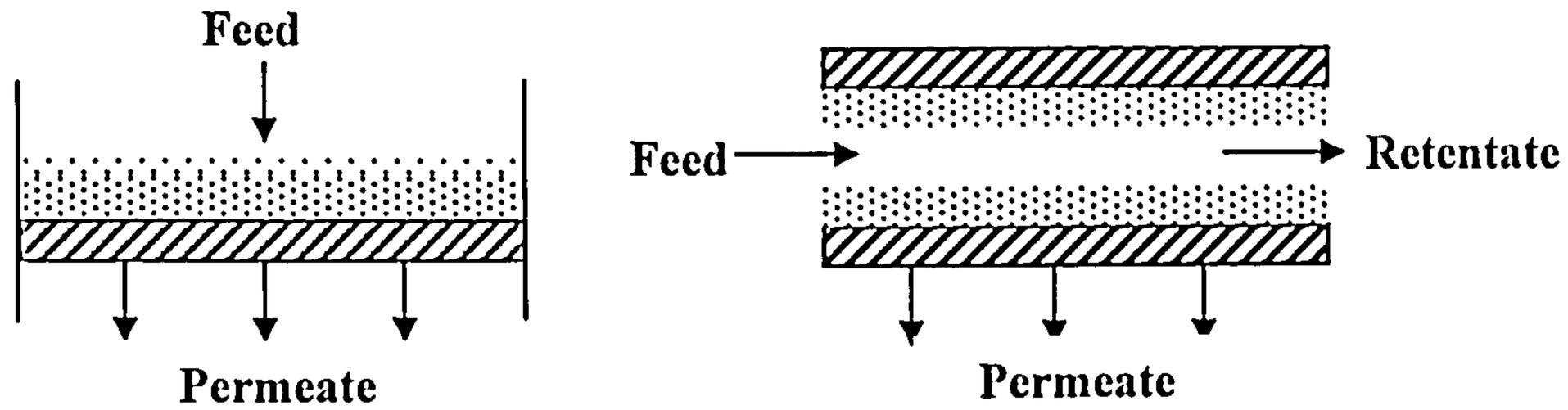
Membrane process can be operated in two modes:

i. dead-end filtration,

ii. cross-flow filtration.

In dead-end or static filtration the solid/liquid suspension is presented to the membrane at right angles to its surface. As separation occurs, the retained solids

build up into a layer which causes resistance to the permeation process. The solids may move back into the bulk of the solution by natural diffusion and when this back transport rate counterbalances the rate of solids being deposited on the membrane a concentration profile is established at the membrane. This phenomenon is commonly referred to as concentration polarisation. In cross-flow separation, the liquid suspension is recirculated in a direction parallel to the membrane surface so that liquid shear tends to sweep away any accumulated solids thereby improving the rate of filtration.



DEAD-END **CROSS-FLOW**

Figure 2.6: Schematic representation of dead-end and cross-flow mode filtration (adapted from Stephenson et al., 2000).

Table 2.3: AMBr applications for domestic and municipal wastewater treatment (adapted from Cicek, 2003).

Source wastewater operation type	Membrane configuration	Size of operation	Treatment success	Country of application	References
Wool scouring	Ultrafiltration external	Pilot-scale ~ 10 m^3/d	TOD removal >89%	Japan	Hogetsu et al. 1992
Pulp mill	Ultrafiltration external	Pilot-scale ~ 10 m^3/d	TOC removal >85%	Japan	Minami 1994

Kraft pulp mill	Ultrafiltration external	Bench-scale 0.003 m^3/d	TOC removal >93%	Canada	Berube and Hall 2001
Dairy whey	Ultrafiltration external	Pilot-scale 0.46 m^3/d	COD removal >94%	USA	Sutton et al. 1996
Maize/egg processing	Ultrafiltration external	Full-scale 500 m^3/d	COD removal >97%	South Africa	Ross et al. 1992
Brewery effluent	Ultrafiltration external	Pilot-scale ~ 10 m^3/d	TOC removal >97%	South Africa	Strohwald and Ross 1992
Liquor production	Ultrafiltration external	Pilot-scale ~ 1.25 m^3/d	COD removal >98%	Japan	Nagano et al. 1992
Sludge production	Microfiltration external	Pilot-scale 0.13 m^3/d	not available	South Africa	Pillay et al. 1994
Palm oil mill	Ultrafiltration external	Bench-scale 0.02 m^3/d	COD removal >93%	Malaysia	Fakhru'l-Razi and Noor 1999
Synthetic wastewater	Microfiltration external	Bench-scale 0.013 m^3/d	COD removal >96%	France	Cadi et al. 1994
Brewery wastewater	Ultrafiltration external	Pilot-scale 0.048 m^3/d	COD removal >99%	England	Ince et al. 2000
Synthetic	Ultrafiltration submerged	Bench-scale	COD removal >90%	England	Hu and Stuckey 2006

2.7 HERBICIDES

There are three major groups of herbicides, namely phenoxy acids, phenylurea and triazine, which are mostly used either for pre- or post-emergence weed control in agricultural crops (Table 2.4). However among these three groups the phenoxy acids are the most widely used globally (Brüsch and Felding, 2000; Aspelin, 1997; Tuxen, et al., 2006).

Table 2.4: Groups of herbicides and their applications.

Groups of Herbicides	Examples of Herbicides	Usage
Phenoxy acids	MCPP, 2,4-D, MCPA, dichlorprop, 2,4,5,-T	control of broad-leaved weed in cereal crops and lawn (Zipper et al., 1996)
Phenylurea	isoproturon, diuron, monuron, linuron, fenuron	weed control on non-crop area and as selective pre-emergence on crops as citrus, asparagus, bush fruits, etc (Peňa, et al., 2002)
Triazine	atrazine, terbutryn, simazine, terbuthylazine	control of the broad-leaved weeds in corn, soybean, peanuts, potato, garlic, orchard and mulberry fields (Kodama, et al., 2001)

All phenoxy herbicides have a very similar chemical structure, comprising a benzene ring with a carboxylic acid side chain of varying length attached, and a varying number of chlorine and/or methyl groups bonded directly to the benzene ring, and of these the chlorinated phenoxyalkanoic acid herbicides are most common (Thomson, 1982). In New Zealand, 68% of all herbicides applied are phenoxy herbicides (Holland and Anis, 1999).

2.8 (*RS*)-MCPP

Mecoprop ((*RS*)-2-(2-methyl-4-chlrophenoxy)-propionic acid) or (*RS*)-MCPP is a herbicide categorized under chlorophenoxyalkanoic or phenoxy acids. It is a white to light brown solid with chemical formula of $C_{10}H_{11}ClO_3$. The chemical structure of (*RS*)-MCPP is shown in Figure 2.6 and as a carboxylic acid, the

molecule will be ionised at neutral and alkaline pH. The presence of an asymmetric (chiral) carbon atom in the aliphatic side chain results in two different optically active forms (stereoisomers or enantiomers), the R-isomer and the S-isomer (Williams et al., 2003). (*RS*)-MCPP herbicides comprise equal proportions of the R- and S- isomers i.e. a racemic mixture.

One of the most important physical properties of (*RS*)-MCPP is its solubility, because this controls (*RS*)-MCPP's transport and fate in the surrounding environment. (*RS*)-MCPP is very soluble in water, 620 $mg.L^{-1}$ at 20 °C (Howard and Meylan, 1997), which promotes its entry into surface or ground waters by natural drainage or infiltration (Gerecke et al, 2002). (*RS*)-MCPP was first identified as a herbicide in 1953 (Tomlin, 1997) and introduced commercially in 1956 (Smith, 1989). According to Department of Environment (1994a), (*RS*)-MCPP was the fourth most widely used herbicide active ingredient used on arable crops in England and Wales in 1990.

Davis et al., (1990) reported that 4000 tonnes of (*RS*)-MCPP annual application rate in the UK and is most commonly applied in formulations as a salt (Fletcher et al., 1995). Its application not only restricts weed growth but also acts as an algicide in paints and coatings and roof protection agents. According to Bucheli et al., (1998), (*RS*)-MCPP was detected at concentrations up to 500 $\mu g.L^{-1}$ in the runoff from roofs that have been treated with Preventol B2, a bi-ester of (*RS*)-MCPP. Furthermore, they suggested that the contamination load from the roof run-off was in the same order of magnitude as the load generated by agricultural applications.

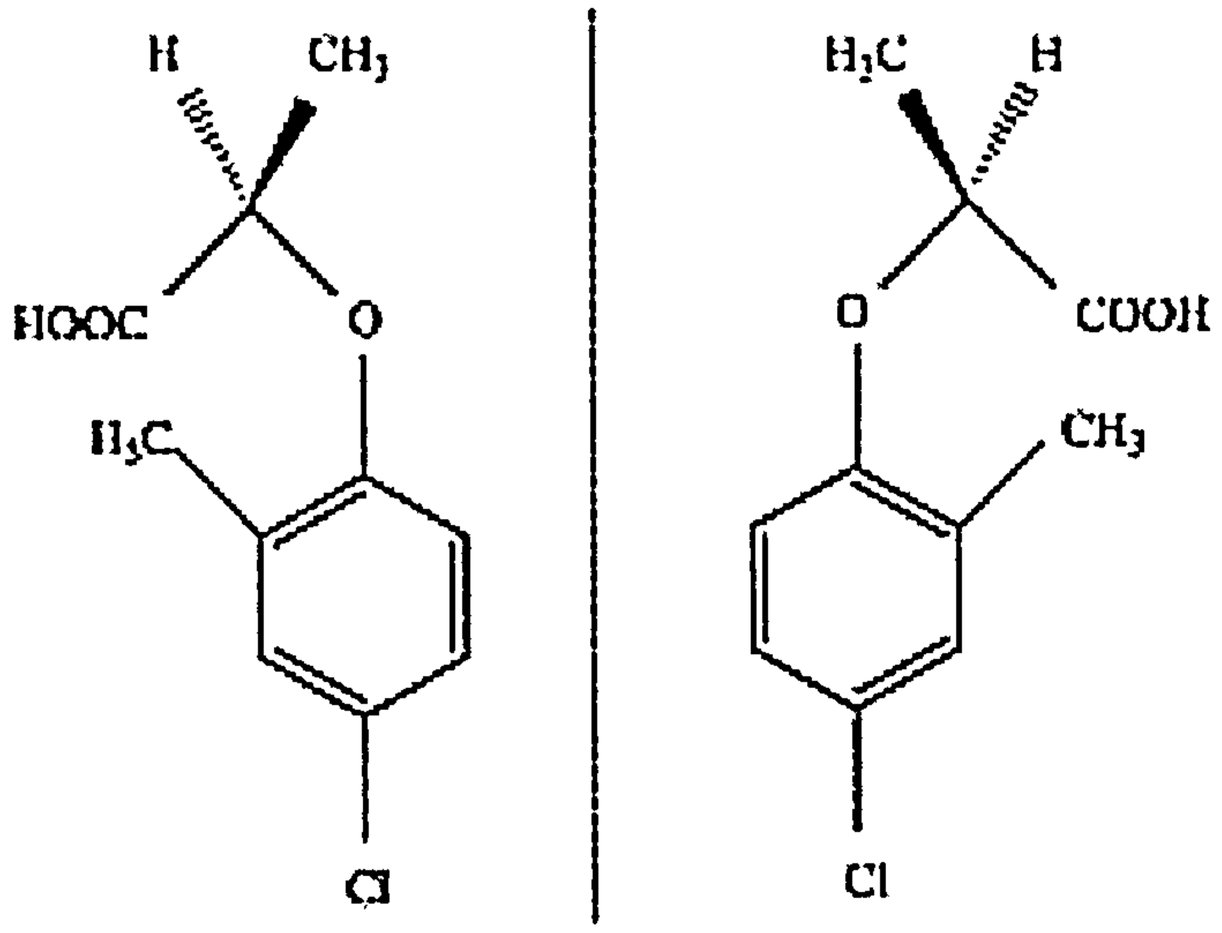


Figure 2.7: Molecule structure of MCPP showing the two enantiomers (Williams et al., 2003).

2.8.1 Possible Pathways for Contamination

Phenoxy acid herbicides generally, such as (*RS*)-MCPP, are released into the environment via two main sources:

i. Surface application

ii. Landfill leachate

2.8.1.1 Surface Application

Herbicides are applied to a variety of terrain, mainly on agricultural land, golf courses, recreation area, lawn and turf production and railways tracks and siding. According to Gerecke et al., (2002), surface application in rural areas is the largest diffuse source of herbicides. Point sources of release include accidental spills, disposal of unused product, runoff from flat roofs and manufacturing

wastewater. Agricultural applications may also lead to herbicide contamination of surface water, sewerage and septic tanks if not performed properly.

2.8.1.2 Landfill Leachate

Accumulation of herbicides in landfills is mainly from the disposal of material related to herbicides such as plant residual, household products that contain herbicides, herbicide packaging, sludge and manufacturing waste. Gintautas et al.; (1992) reported that several phenoxy herbicides have been identified in the leachate from six municipal landfill sites in the US, and (*RS*)-MCPP was consistently present in all samples.

In Denmark, (*RS*)-MCPP concentrations up to 250 $\mu g.L^{-1}$ have been identified in an anaerobic leachate plume (Lyngkilde & Christensen, 1992) while a concentration of 39 $mg.L^{-1}$ has been recorded around a landfill in Lincolnshire, UK which was responsible for contamination of an aquifer used for drinking water abstraction (Harrison et al., 1998). A monitoring programme of private shallow bore-holes in the Triassic Sherwood Sandstone aquifer of South Yorkshire, UK showed that 34 % of the 14 bore-holes investigated contained concentrations of herbicides in excess of the EU permissible limit and (*RS*)-MCPP was one of the most commonly identified (Lapworth et al., 2006).

2.9 (*RS*)-MCPP BIODEGRADATION IN ENVIRONMENT

Laboratory studies conducted using microcosm found that (*RS*)-MCPP was more biodegradable under aerobic than in anaerobic conditions (Harrison et al, 2003) with both enantiomeric forms able to degrade under this condition.

In addition, Harrison et al, 2003 reported that under nitrate-reducing microcosms, only (*R*)-MCPP could be biodegraded under anaerobic conditions but not (*S*)-MCPP as its concentration remained constant showing no signs of biodegradation as shown in Figure 2.8.

4-chloro-2-methylphenol was detected as intermediate product from (*RS*)-MCPP degradation. The concentration of 4-chloro-2-methylphenol only starts to drop once the (*R*)-mecoprop has fully degraded. The production of this product is of high concern as it contains acute toxicity to fish if it is released to surrounding waters (Harrison et al, 2003).

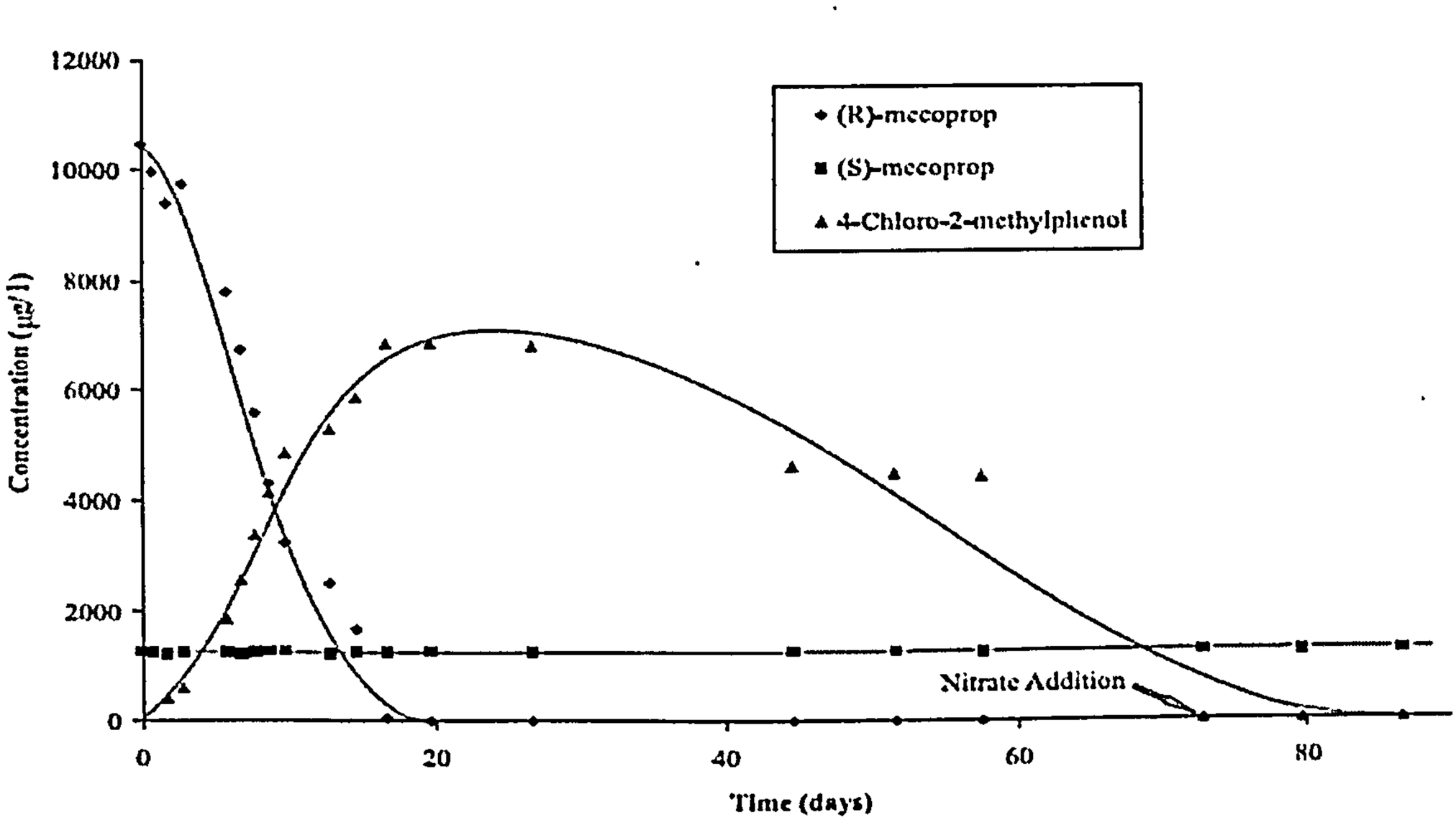


Figure 2.8: Biodegradation of (*R*) and (*S*)-mecoprop under anaerobic conditions (taken from Harrison et al, 2003).

Under aerobic conditions (Figure 2.9), (*S*)-MCPP was found to degrade faster and no toxic by-products are formed from either of the enantiomeric forms of

(*RS*)-MCPP. This makes it more favourable than anaerobic biodegradation. It is also found that when comparing anaerobic with aerobic conditions, the latter achieved a faster degradation of both enantiomeric forms of MCPP.

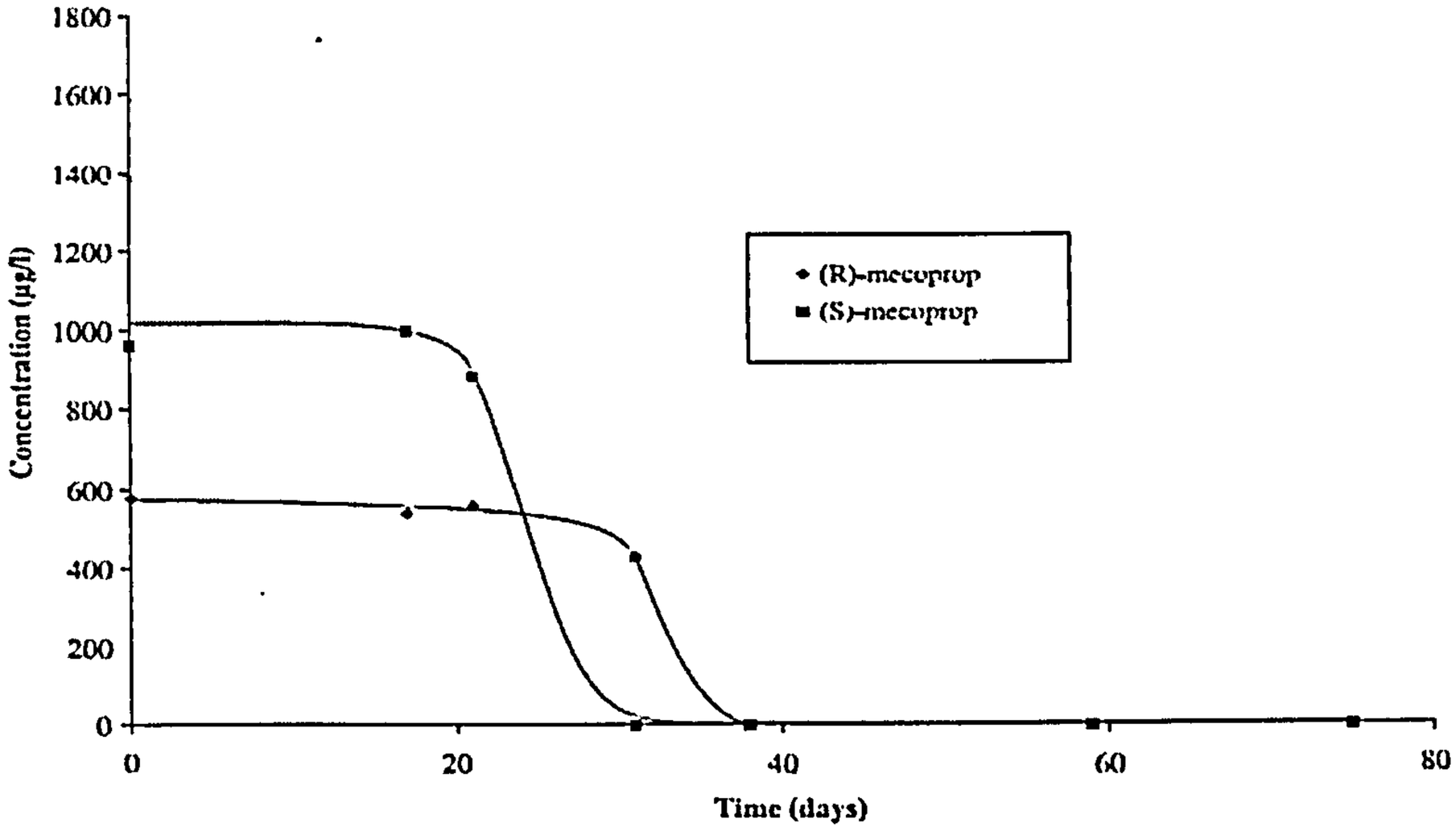


Figure 2.9: Biodegradation of (*R*) and (*S*)-mecoprop under aerobic conditions (taken from Harrison et al, 2003).

A study by Nitschke et al, (1999) revealed that high adaptation period needed in laboratory activated sludge plants before successful biodegradation occurred (Figure 2.10). This adaptation period is known as a lag phase (Nitschke et al, 1999). As a result, during the lag phase little or no removal of (*RS*)-MCPP was achieved. This can be seen in Figure 2.9 where the concentration remained constant or in Figure 2.10 where the percentage removal was minimal. The environmental factors that influence the degradation rate of (*RS*)-MCPP in groundwater and soil are discussed below.

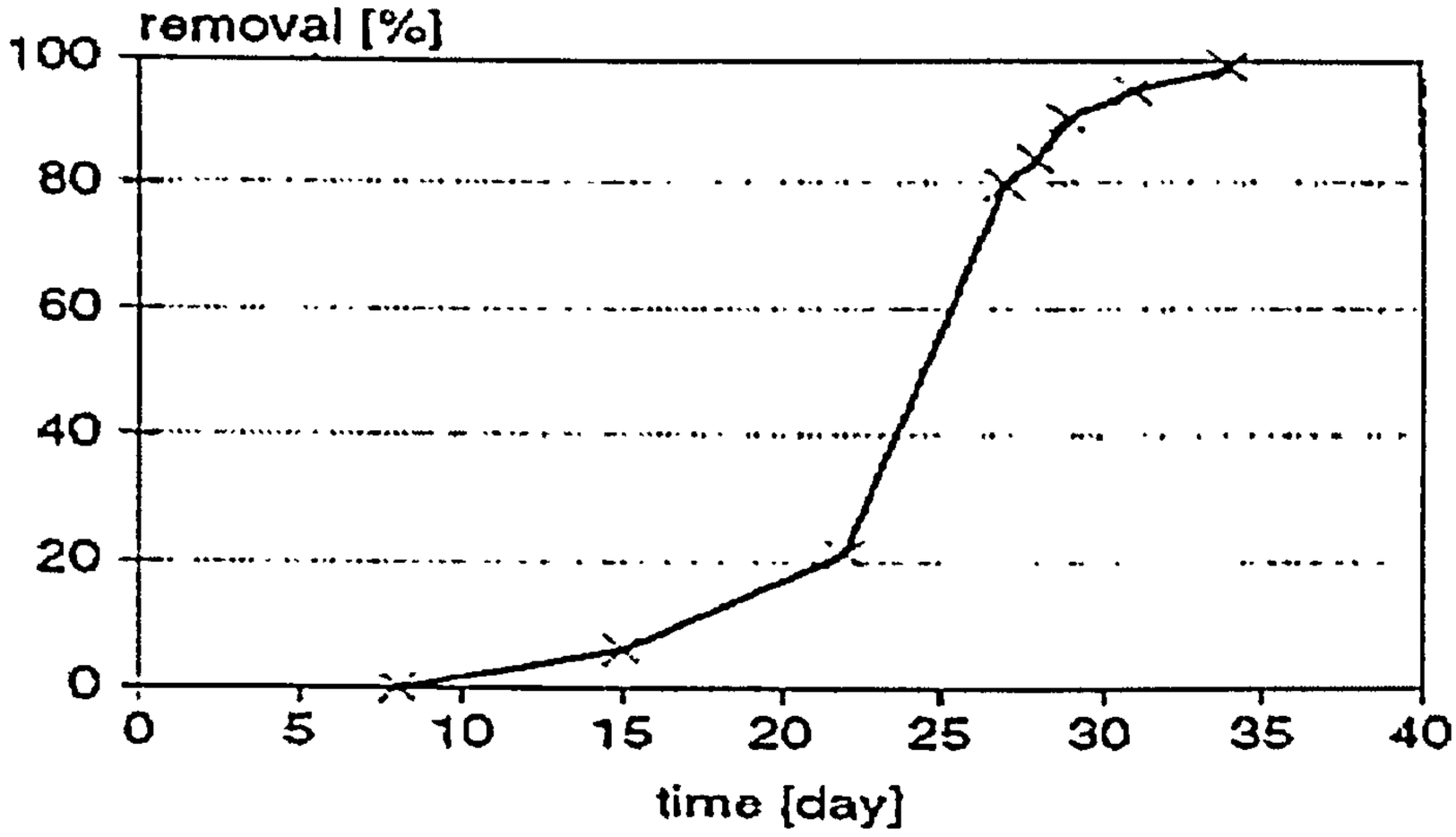


Figure 2.10: Removal of mecoprop in laboratory activated sludge units (taken from Nitschke et al, 1999).

2.9.1 Redox Conditions

Degradation tests are usually reported under aerobic conditions but limited degradation has been observed in nitrate-reducing environments (Larsen and Aamand 2001; Tuxen et al. 2003; Harrison et al., 2003; Reitzal et al., 2004). Harrison et al., (2003) reported that only the *R*-isomer was degraded under nitrate-reducing conditions.

2.9.2 pH

Johnson et al., (2003) in their work within aerobic UK aquifer systems have hypothesised that (*RS*)-MCPP degradation in groundwater may be dependent on low pH but later found that (*RS*)-MCPP could be degraded at pH around 7.7 suggesting that low pH is not an obligatory factor for (*RS*)-MCPP degradation.

2.9.3 Exposure to (*RS*)-MCPP

Harrison et al., (1998) in their study of phenoxyacid herbicides in a limestone aquifer noted that lag times in aerobic microcosms were much shorter than other studies in previously unpolluted aquifers. This finding was supported by Torang et al., (2003) who found that previous exposure of the aquifer to (*RS*)-MCPP resulted in reduced or no lag time before the onset of rapid degradation. Furthermore, they concluded that the amount of previous exposure determined the effect of lag time on degradation, for example no lag time being apparent for aquifer sediments previously exposed to (*RS*)-MCPP at concentrations greater than 100 $\mu g.L^{-1}$. In contrast, a lag time was observed in aquifer sediments previously exposed to between 10 and 100 $\mu g.L^{-1}$.

2.9.4 Oxygen Concentration

In laboratory studies, Rugge et al., (2002) found decreased lag times and increased aerobic degradation rates with higher oxygen concentrations and this finding is in parallel with those of Rietzel et al., (2004).

2.9.5 (*RS*)-MCPP Concentration

(*RS*)-MCPP concentration plays a vital role in the rate of degradation (Agertved et al., 1992; Albrechtsen et al., 2001). Studies on the effect of concentration by Torang et al., (2003) have been hampered by difficulties in reproducing (*RS*)-MCPP degradation in previously unexposed aquifer samples at concentrations between 0.025 $\mu g.L^{-1}$ and 100 $\mu g.L^{-1}$.

However, for pre-exposed samples they managed to observe that (*RS*)-MCPP degradation at concentrations below 10 $\mu g.L^{-1}$followed first order non-growth kinetics. In contrast, at concentration above 10 $\mu g.L^{-1}$ the biodegradation rate accelerated gradually due to selective growth of specific biomass. Furthermore, the enhanced rate of degradation by adapted systems was maintained throughout degradation, even at concentrations below 0.1 $\mu g.L^{-1}$. These results suggest the development of metabolic capability is dependent on acclimation and is also sensitive to the concentration of herbicides.

Table 2.5: Studies on (*RS*)-MCPP degradation under different conditions.

References	Condition	(*RS*)-MCPP Concentration	Degradation Rate
Reizel et al., 2004	Field and laboratory microcosms under anoxic conditions	< 1 $\mu g.l^{-1}$ and 15-18 $\mu g.l^{-1}$	Degraded
Baun et al., 2003	Naturally aerobic aquifer, anaerobic conditions in a area of plume downgradient from landfill	26-600 $\mu g.l^{-1}$	No degradation
Harrison et al., 2003	Laboratory microcosms (aerobic, anaerobic and anoxic)	500-10471 $\mu g.l^{-1}$	No degradation under methanogenic/sulphur reducing; 0.65 $mgL^{-1}d^{-1}$ under nitrate reducing; 1.9 $mgL^{-1}d^{-1}$ under aerobic
Williams et al., 2003	Field and laboratory microcosms under different redox conditions	500-10471 $\mu g.l^{-1}$	No degradation in methanogenic/sulphate reducing but degraded under iron, nitrate reducing and aerobic
Tuxen et al., 2003	Field and laboratory (aerobic and anaerobic)	65 $\mu g.l^{-1}$	Aerobic degradation after short lag phase; Anaerobic degradation after long lag phase

Toräng et al., 2003	Laboratory (aerobic)	0.025-100 $\mu g.l^{-1}$	Degradation rates closely linked to previous exposure
Tuxen et al., 2002	Laboratory microcosms (aerobic)	25 $\mu g.l^{-1}$	Variable
Broholm et al., 2001	Aerobic aquifer	40 $\mu g.l^{-1}$	No degradation
Larsen et al., 2000	Laboratory mineralisation studies; (aerobic and anaerobic)	8-71 $\mu g.l^{-1}$	Aerobic-mineralisation observed; Anaerobic-3.3 % of added (*RS*)-MCPP recovered as $^{14}CO_2$ after 312 days
Tett et al., 1997	Batch culture (A. *denitrificans*)	5 $g.l^{-1}$	Degraded

2.10 MOLECULAR ANALYSES OF MICROBIAL COMMUNITIES IN REACTOR SLUDGE

The use of conventional microbiological methods based on isolation of pure cultures and morphological, metabolic, biochemical and genetic assays to study the biodiversity of complex ecosystem in an environmental sample has been shown to be unreliable (Sanz and Köchling, 2007). These methods are of limited use as they are not only time consuming but fail to give a representative picture of the bacterial diversity of an ecosystem. Direct microscopic counts generally exceed the number of colony forming units (CFU) by several orders of magnitude (Statley and Konopka, 1985).

Moreover, it has been estimated that more than 99 % of microorganisms observable in nature typically are not cultivated by standard techniques (Hugenholtz et al., 1998). These problems are exacerbated in studies of fastidious anaerobes because of their low growth rates and oxygen sensitivity

(obligate anaerobiosis), and methanogens are among the most difficult microorganisms to study by culture-based techniques (Bell, 2002). One solution to these problems is to use molecular biology approaches such as ribosomal RNA and DNA analysis by fluorescent *in situ* hybridisation (FISH), denaturing gradient gel electrophoresis (DGGE) and single strand conformation polymorphism (SSCP).

2.10.1 Fluorescent Staining Using DAPI

DAPI (4',6-diamido-2-phenylindole) is a fluorescent dye used to stain microorganisms in opaque habitats. Cells that are stained with DAPI fluoresce are bright blue and easy to see and enumerate. It has the advantage of being non-specific and detects all microorganisms in a sample. However, one of the drawbacks in this staining technique is it fails to differentiate between living and dead cells or between different species and thus cannot track specific organisms in an environment (Madigan and Martinko, 2006). In this study, DAPI staining was carried out before fluorescent *in situ* hybridization (FISH) to determine the volume of anaerobic sludge samples containing 10^8 permeabilized cells required for hybridisation. The method of total cell counts using DAPI has been described in detail elsewhere (Kepner and Pratt, 1994; Davenport et al., 2004).

2.10.2 Fluorescent *in situ* hybridisation (FISH)

Fluorescent *in situ* hybridization (FISH) technology is widely used in microbial ecology and clinical diagnostics. The principle of FISH is based on the tendency of labelled cellular ribosomal RNA to bind specifically to its complementary

sequences (hybridize) in the target cells; thus the cells become uniformly fluorescent and can be observed under a fluorescent microscope. There are several significant advantages of FISH as outlined below:

i. FISH coupled with confocal laser microscopy (CLSM) can be used to obtain spatial and three dimensional distribution of cells *in-situ* without requirement for mechanical sectioning of the samples (Wagner et al., 1998);

ii. FISH using rRNA-targeted oligonucleotide probes facilitates the rapid and specific identification of individual microbial cells in their natural environments without prior cultivation, and has been successfully used to detect and identify uncultured bacteria (DeLong et al., 1989; Amann et al., 1990a, 1990b, 1995; Wagner et al., 1993; Manz et al., 1994);

iii. FISH can be used as a quantitative method, which allows enumeration of individual cells and provides information about the abundance of the target organism (Wegner et al., 1998);

iv. Easy and fast if suitable probes are available and allow direct visualization of non-cultured microorganisms.

Despite being an increasingly popular technique and widely used in microbial ecology and clinical diagnostics, this technique still has limitations. The problems associated with FISH methods have been widely discussed and reviewed elsewhere (Coskunar, 2000; Dabert et al., 2002 and Bouvier and del Giorgio, 2003). The limitations are as outlined below:

i. Cell permeability to the probes, gram-negative bacteria are sufficiently permeabilized but certain Gram-positive bacteria are not (Manz et al.,

1993; De Los Reyes, 1997) and may require enzymatic treatment (Davenport, 2000);

ii. Fluorescent signal may be absent because of the presence of small numbers of cells or low rRNA content within the cells (less than 10^3 to 10^4 cells per mL (Aman et al., 1995);

iii. Not all sites within the ribosome are equally accessible for FISH, as they may be blocked by rRNA structure (Che Man, 2006).

2.10.3 Confocal Scanning Laser Microscope (CLSM)

The fluorescent stains that are used to quantify the relative abundance of specific microorganisms based on fluorescent *in situ* hybridization (FISH) (Daims et al., 2001; Juretschko et al., 2002; Mantz et al., 1998; Schmid et al., 2003) can be visualised by CLSM. CLSM images can be acquired which have sharper images than conventional microscopy, and the method allows for an optical sectioning, as opposed to a physical sectioning (Li and Ganczarczyk, 1991), which can adversely affect the floc structure and has important limitations on the size of the aggregates that can be analysed.

CLSM has many distinctive advantages compared with conventional miscroscopy such as epifluorescent microscopy. Some of the advantages are as follows;

i. rejecting of signals emanating from out of focus regions;

ii. elimination of background due to light scattering by the specimen;

iii. optimization of "signal to background" and "signal to noise ratio";

iv. detection of back scattered photons by optical inhomogeneities (e.g. enzymatic reaction products) in a non interfering manner;

v. quantitative approach to imaging optical probes (e.g. fluorescence intensity is only related to the intracellular concentration and brigtness of the probe);

vi. enhancement of resolution by a factor of 1.4 due to pinhole and objective taking part equally in image formation.

The most important advantage of CLSM is that it offers three dimensional imaging, by a technique known as optical sectioning. Stacks of optical sections taken at successive focal planes known as z series can be reconstructed to produce a three dimensional view of the specimen (Matsumoto, 1993) which is paramount to view biological objects such as cells and tissues.

2.11 SUMMARY OF LITERATURE REVIEW

From the literature review it can be concluded that:

Under certain conditions, (*RS*)-MCPP May degrade slowly in the environment:

- (*RS*)-MCPP less biodegradable under anaerobic than aerobic conditions;
- only a basic understanding exists of (*RS*)-MCPP biodegradable under anaerobic conditions with different electron acceptors;
- under nitrate-reducing microcosms, only (*R*)-MCPP could be biodegraded under anaerobic conditions (not (*S*)-MCPP);
- no research has been carried out on biodegradability of (*RS*)-MCPP in an AMBr system;

- there is only a basic understanding of factors that influence the metabolic capability or degradation rate of (*RS*)-MCPP under different redox conditions.

Advantages of the AMBr configuration for wastewater treatment plant:

- HRT is independent of SRT;
- retains the majority of biomass within the reactor;
- operates as a high rate processes due to good mixing and high biomass concentrations;
- reduces footprint and operational cost compared to conventional treatment plants;
- can produce high quality effluent which can meet stringent environmental discharge requirements.

An AMBr treating wastewater contaminated with (*RS*)-MCPP will:

- be able to degrade (*RS*)-MCPP more effectively by encouraging a diverse anaerobic microbial population to develop;
- contain the majority of the biomass within the reactor and thus increase biomass acclimatisation towards (*RS*)-MCPP;
- increase process stability (via high SRT) towards elevated concentrations of (*RS*)-MCPP in wastewaters (concentration pertubations in the effluent);
- improve our understanding of the anaerobic degradation (*RS*)-MCPP in wastewaters containing high levels of this compound;
- provide a significant understanding in terms of interaction between denitrifiers, or sulphate reducing bacteria, with methanogens during the

anaerobic degradation (*RS*)-MCPP in wastewaters containing alternative terminal electron acceptors such as nitrate and sulphate (i.e. anions that may be present at significant concentrations in the wastewaters from herbicide manufacturing plants).

CHAPTER THREE

AIM & OBJECTIVES

3.1 AIM

The aim of this research was to investigate the characteristics of an anaerobic membrane bioreactor (AMBr) operating with a range of different electron acceptors during the anaerobic treatment of wastewater containing mecoprop ((*RS*)-MCPP), and to identify whether the separation of Hydraulic Retention Time (HRT) and Solid Retention Time (SRT) in the AMBr could promote the growth and retention of bacteria involved in the degradation of (*RS*)-MCPP. Therefore, the scope of study in this research will be established based on the all factors described above.

3.2 OBJECTIVES

Several objectives were set in order to achieve the aim of the study, they were:

i. to develop a method using High Performance Liquid Chromatography (HPLC) for the analysis of (*RS*)-MCPP in aqueous samples that could be used throughout the study on effluent samples;

ii. to investigate the toxicity of (*RS*)-MCPP on process performance of the AMBr and identify the specific (*RS*)-MCPP utilisation characteristics at elevated (*RS*)-MCPP concentration under methanogenic conditions;

iii. to investigate (*RS*)-MCPP degradation in the AMBr in the presence of nitrate as an available terminal electron acceptor, and to assess the influence of the

COD/N-NO_3^- ratio on methanogenic and denitrifying activities in a single reactor unit;

iv. to investigate (*RS*)-MCPP degradation in the AMBr in the presence of sulphate as an available terminal electron acceptor, and to assess the influence of the COD/SO_4^{-2-} ratio on methanogenic and sulphate reducing activities in a single reactor unit;

v. to evaluate the effects of step changes in HRT and OLR on the process performance and (*RS*)-MCPP degradation efficiency of the AMBr, under methanogenic conditions, with the hypothesis that long HRT provides sufficient contact time with biomass for uptake and degradation;

vi. to investigate the effect of trace levels of oxygen in the headspace of the effluent tank on (*RS*)-MCPP degradation;

vii. to investigate, how different terminal electron acceptors affect the microbial populations present in the AMBr sludge, and identify whether any link exists with key trophic groups present in the reactor.

CHAPTER FOUR

MATERIALS AND METHODS

4.1 INTRODUCTION

Chapter four describes the materials and methods used and other methodology related to this work. This chapter also describes the preliminary work carried out during the method development using HPLC for analyzing (*RS)*-MCPP contained in permeate and biomass samples in the AMBr. Initially, for HPLC method development, all important information related to (*RS)*-MCPP such as physical and chemical properties is gathered and existing analytical methods reviewed in order to identify the best choice of initial conditions for the HPLC method development. The laboratory reactor system used in this study will be presented in detail and a procedure for starting the reactor will be explained. Finally, all standard methodologies for chemical analysis of reactor performance used in this study will be described.

4.2 EXPERIMENTAL DESIGN

The study was carried out in five phases which are shown in a flowchart (Figure 4.1). An initial phase (Phase I) investigated the effect of elevated (*RS)*-MCPP on AMBr under methanogenic conditions. During Phase I, the reactor was operated with a constant organic loading rate (OLR) of around 1.5 kg COD $m^{-3} \cdot d^{-1}$ and a hydraulic retention time (HRT) of 3.5 days.

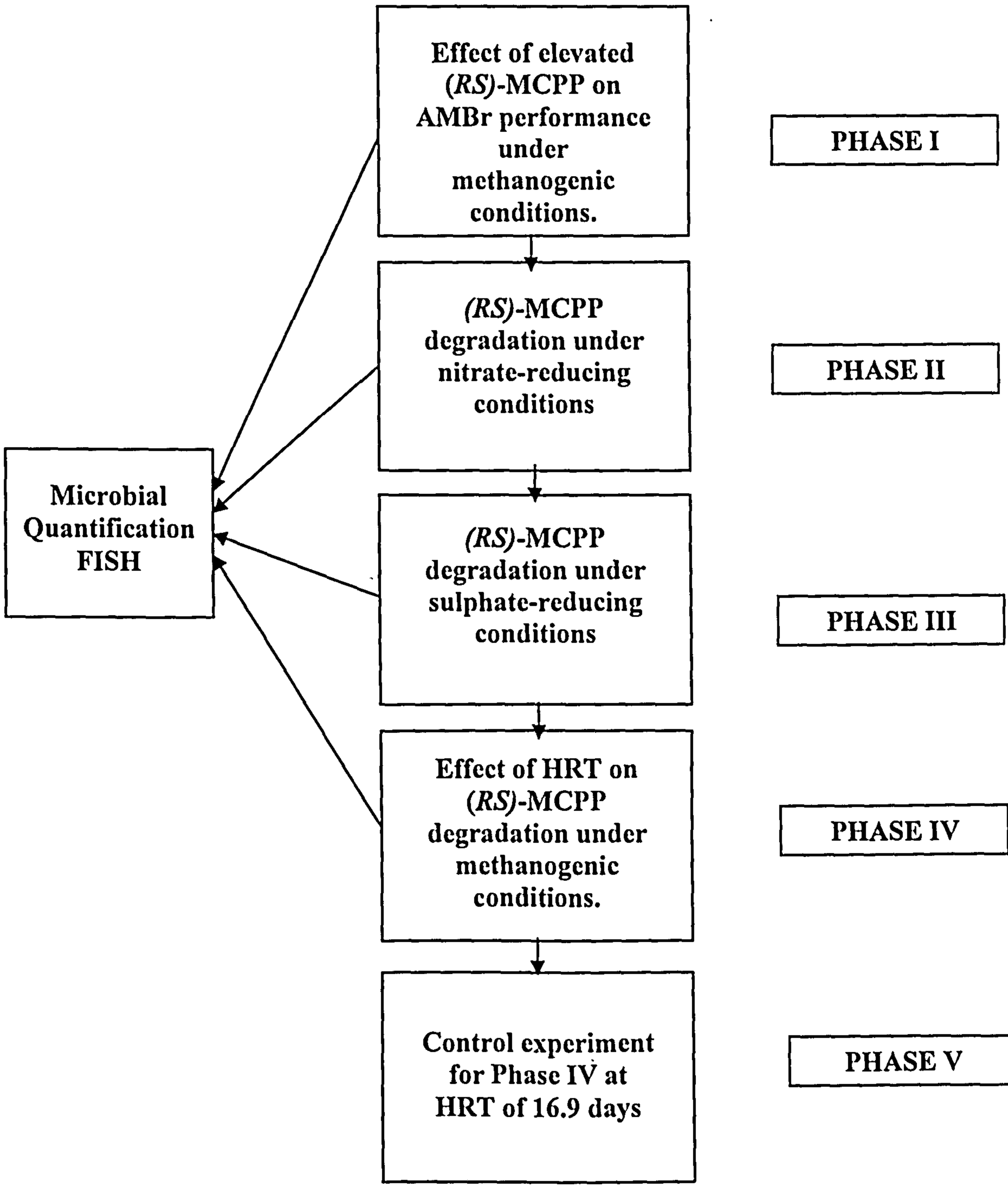


Figure 4.1: Experimental Plan.

Phase II investigated the effect of denitrification on *(RS)*-MCPP degradation. Six different COD to nitrate ratios (COD/NO_3^--N) were investigated; 250, 8, 3, 1, 0.3 and 0.2. During Phase II, the reactor was operated with a constant HRT of 3.3 d and *(RS)*-MCPP concentration of 200 and 100 $mg.L^{-1}$.

In Phase III, the ability of sulphate-reducing bacteria (SRB) to degrade (*RS*)-MCPP was assessed by adding potassium sulphate to support sulphate-reducing conditions. Three different COD to sulphate ratios (COD/SO_4^{2-}) were investigated; 2, 0.4 and 0.2. The reactor was operated at a constant organic loading rate (OLR) of 0.07 $kgCOD.m^{-3}.d^{-1}$, hydraulic retention time (HRT) of 3.3 days and (*RS*)-MCPP concentration of 50 $mg.L^{-1}$.

Phase IV investigated the effect of increasing HRT on the kinetics of (*RS*)-MCPP degradation by the system. (*RS*)-MCPP concentration in the feed was set to 50 $mg.L^{-1}$ throughout this phase. Three different HRT were investigated; 3.4, 6.8, and 16.9 days, giving corresponding OLRs of 0.47, 0.21 and 0.13 $kgCOD.m^{-3}.d^{-1}$. The robustness and stability of the AMBr was investigated in response to an additional hydraulic shock load on the biomass imposed by a rapid increase in flow rate to 20 times that at an HRT of 16.9 days (i.e. HRT was 0.8 days) and maintaining this flow over a period of three weeks before the HRT was increased back to 3.3 days to investigate the recovery of the biomass.

Phase V was a control experiment for Phase IV to further investigate the effect of trace levels of oxygen that would have been introduced by permeate recycling from the effluent tank in Phase IV. In this phase, the same operating conditions

as in Phase IV at HRT 16.9 days were maintained but the effluent tank was made strictly anaerobic providing a nitrogen gas overpressure (see Section 8.2). The experimental design of each phase will be addressed in more detail in each discussion chapter.

4.3 EXPERIMENTAL APPARATUS

4.3.1 Anaerobic Membrane Bioreactor (AMBr)

A schematic representation of the laboratory-scale AMBr is shown in Figure 4.2. The reactor which was made from PVC had a working volume of 18.5 litres and was coupled to a modified Polyethersulphone (PES) hollow fibre membrane filtration unit that has a surface area of 0.2 m^2 and a nominal pore size of 0.5 µm, (Milleniumpore, Washington, UK). The Polyethersulphone (PES) hollow fibre membrane was modified by ozone treatment followed by graft polymerization with 2-hydroxy-ethyl methacrylate (HEMA). Therefore, grafting with HEMA modified the surface properties of the PES membrane, converting it from strongly hydrophobic to hydrophilic; hence reducing membrane fouling caused by hydrophobic adsorption. The fixed volume in the reactor was maintained by a level controller containing three level control probes connected to the recycling pump.

Three probes were used instead of two to provide a time delay to the recycling pump after it received a signal from the level controller, thus preventing damage from frequent switching of the permeate pump. A centrifugal pump, 230 V (Totton Pump Limited, Southampton, UK) recirculated the reactor MLVSS

through the membrane to give crossflow velocity of 3 ms^{-1}. The reactor wall was wrapped with a tubular PVC water jacket (ID 15 mm) to maintain reactor temperature within the range of 36±1°C. Two pressure gauges and a differential pressure gauge measured the pressure inside and outside the membrane filtration unit as well as the pressure differential across the membrane. A solenoid valve was used to produce a periodic backpressure pulse within the membrane filtration unit to help reduce membrane fouling. In this way, the membrane could be operated continuously for 741 days without the need for any physical or chemical cleaning. Effluent was collected in a 50 litre effluent tank via a permeate line. Biogas was collected via a gas line from the reactor headplate to a Dreschel bottle before volume measurement by an optical bubble counter (Chelliapan *et al.*, 2006). Further details of the nitrogen overpressure system (Phase V) are given in Section 8.2.

4.3.2 (*RS*)-MCPP Stability Test

The chemical stability of the synthetic (RS)-MCPP wastewater used in this research was tested by decanting a sample of the synthetic (*RS*)-MCPP wastewater into two sterile sealed Universal bottles. One bottle was placed in a water bath (Grant, VF Grant Instruments Ltd. England) and the other (control) used to determine initial (*RS*)-MCPP content by HPLC analysis. The incubated sample was maintained at the same operating condition as the AMBr, 35 ± 2°C, for a period of 3 weeks before determining residual (*RS*)-MCPP concentration.

4.4 FEED AND NUTRIENTS

The feed used throughout the study was a synthetic (*RS*)-MCPP wastewater containing the desired value of a (*RS*)-MCPP stock solution added to diluted brewery wastewater. The stock solution (*RS*)-MCPP was prepared by weighting 50 g of analytical grade of (*RS)*-MCPP to a 1000 ml volumetric flask containing 10 ml of 1 N NaOH. The brewery wastewater was collected from the Scottish-Newcastle brewery in Newcastle and comprised mainly waste beer, i.e. past its expiry date, and returned beer which was mixed with site process wastewater into a balancing tank; the characteristics are given in Table 4.6 below.

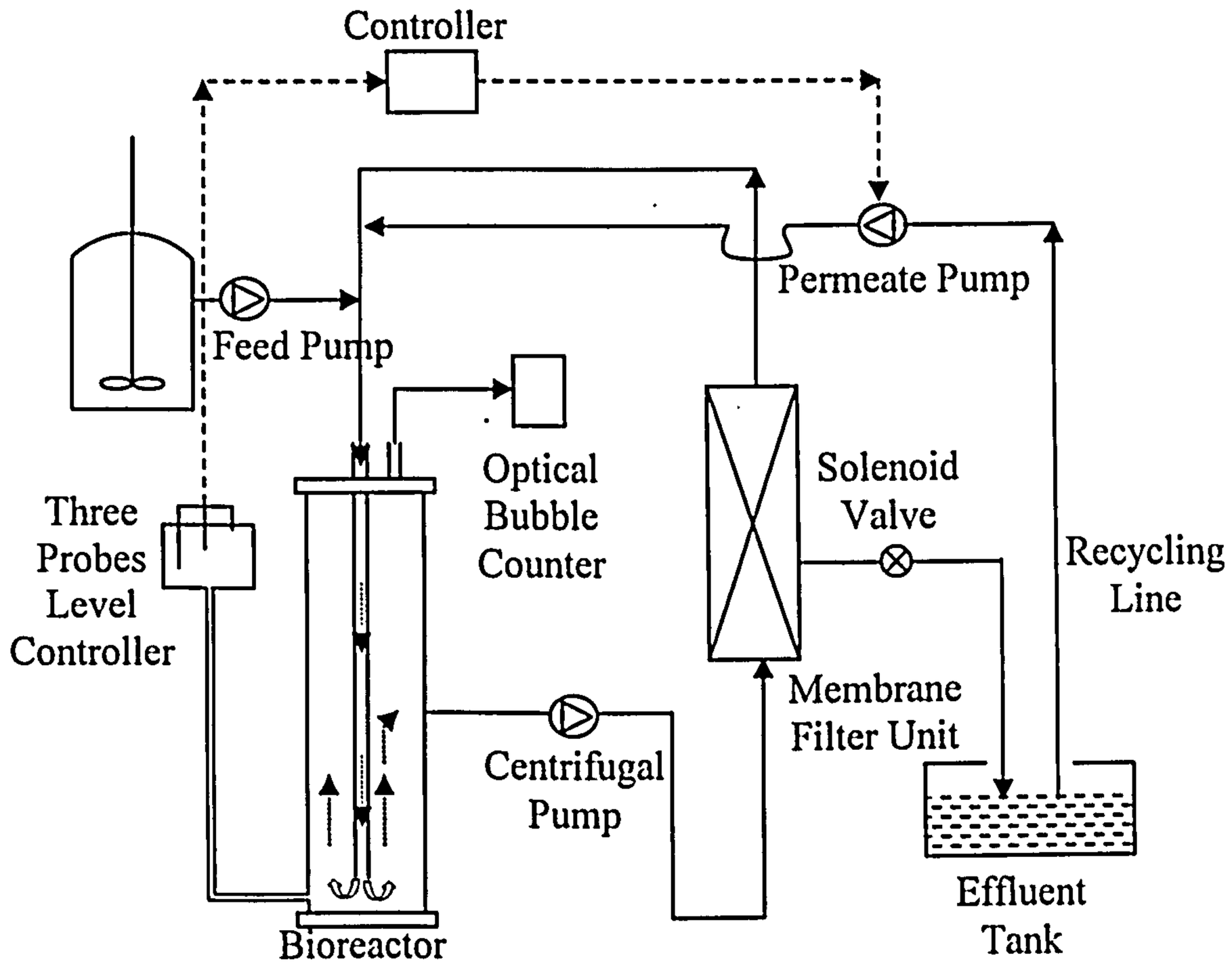


Figure 4.2: Schematic design of the experimental AMBr system.

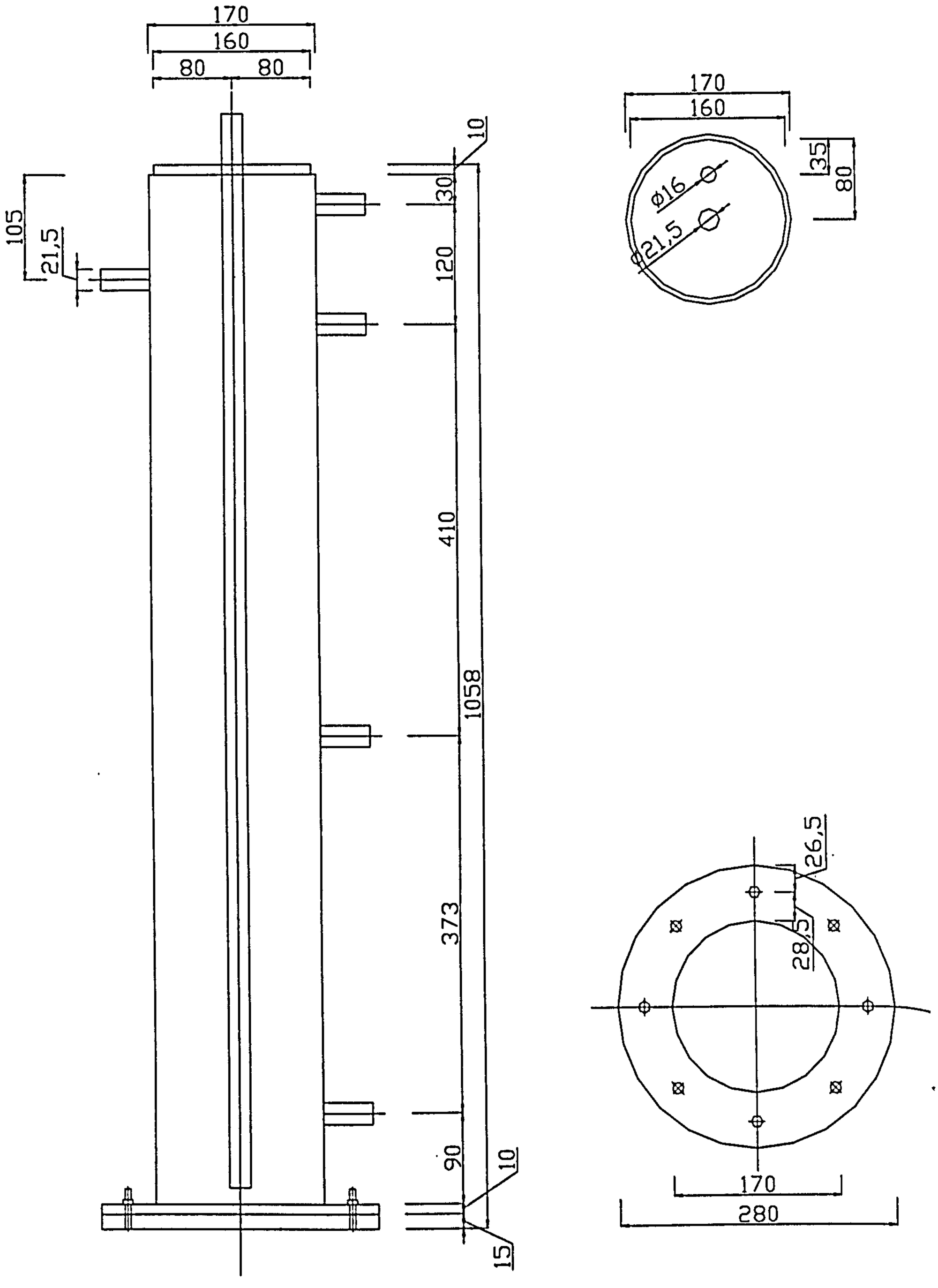


Figure 4.3: Detailed specification of the reactor unit.

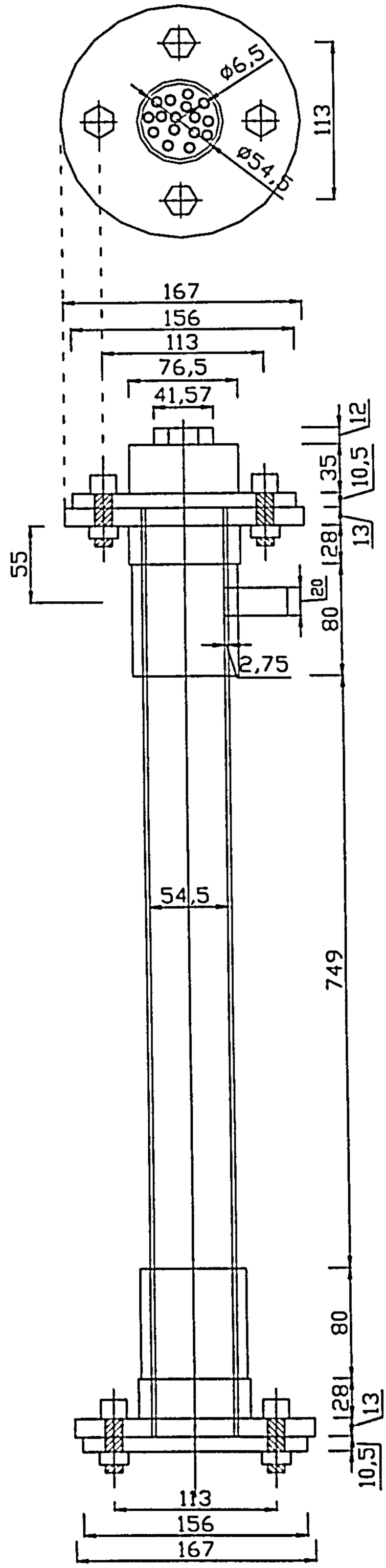


Figure 4.4: Detailed specification of the membrane filtration unit.

Desired COD was obtained by diluting the raw brewery wastewater with tap water and (*RS*)-MCPP stock solution was added to give concentrations of 5-200 $mg.L^{-1}$ (Phase I), 100-200 $mg.L^{-1}$ (Phase II) and 50 $mg.L^{-1}$ (Phase III, IV & V). Throughout the study, the COD : N : P ratio was maintained in the influent at a ratio of 250:5:1. Nitrogen and phosphorus were added to the feed regime in the form of urea ($(NH_2)_2CO$) and KH_2PO_4. A commercial micro-nutrient supplement, Nutromex TEA 310, supplied by OMEX Environmental Ltd containing the following trace elements per litre: 25.7 mg iron, 11.1 mg manganese, 13.1 mg nickel, 12.4 mg cobalt, 1.33 mg zinc, 0.2 mg molybdenum, 0.2 mg copper, 0.004 mg aluminium, 0.044 mg calcium, 0.014 mg magnesium, 30.6 mg sodium and 0.018 mg potassium, was added at 0.01 mL TEA supplement added for each 5000 mg COD to correct for possible trace element deficiency (metal precipitation) of the brewery wastewater under the low redox conditions of all experiments.

In Phase II and III, potassium nitrate and potassium sulphate were added (Section 6.2 and Section 7.2) to the synthetic wastewater to support nitrate-reducing and sulphate-reducing conditions. The trace elements deficiency of the synthetic wastewater was corrected by adding a trace elements solution (COD:N:P = 250:5:1) and nutrient deficiency was corrected by using Nutromex TEA 310.

Table 4.1 The characteristic of brewery wastewater.

Parameter	Concentration ($mg.L^{-1}$)
COD	75,000-80,000
N	220
TKN	420
SO_4^{-2}	200
NO_3^{-1}	n/d
PO_4-P	180
Suspended solids	150-250
pH (units)	3.5-4.5

Table 4.2: Nutrient concentrations in Omex Nutromex TEA 310.

Parameters	Concentration ($mg.L^{-1}$)
Iron	25.7
Manganese	11.1
Nickel	13.1
Cobalt	12.4
Zinc	1.33
Molybdenum	0.2
Copper	0.2
Aluminium	0.004
Calcium	0.044
Magnesium	0.014
Sodium	30.6
Potassium	0.018

4.5 SEEDING THE AMBR REACTOR (COMMISSIONING)

Anaerobic sludge taken from an anaerobic sludge digester at Hexham Municipal sewage treatment plant (Northumberland, UK) was used as seed. This was first screened through a 1-2mm mesh to remove fibres and hair to prevent damage to the centrifugal pump, giving a final solids content of 34,000 mg $TSSL^{-1}$ (22,150 mg $VSSL^{-1}$). After the sludge was screened, 6 L was then introduced into the reactor through the anterior inlet. The remaining volume of the system was filled with tap water to give a final sludge concentration of 7180 $mgVSS.L^{-1}$. After seeding, the anterior inlet was sealed and the reactor was flushed with nitrogen gas to eliminate oxygen and protect the sensitive methanogenic bacteria. Then, the reactor was allowed to stabilize at 37°C for 24 hours prior starting the study.

4.6 SAMPLING AND ANALYSIS

The analyses of chemical oxygen demand (COD), total organic carbon (TOC), pH, alkalinity, volatile suspended solids (VSS), suspended solids (SS) and total Kjeldahl nitrogen (TKN) were carried out in accordance with Standard Methods (APHA 1998). Table 4.8 tabulated types of analysis, frequency and point of sampling carried out during sampling and analysis. Detailed chemical analysis is described briefly below.

4.6.1 Chemical Oxygen Demand (COD)

This test was performed to verify the TOC concentrations and to determine the empirical ratio between the two parameters. This was particularly important should any problem arise with the TOC equipment. Samples were oxidised with

a known excess of potassium dichromate ($K_2Cr_2O_7$) in a closed and strongly acidic environment. Following that, the remaining unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulphate to determine the amount of $K_2Cr_2O_7$ consumed which represents the oxidisable matter in the sample in terms of oxygen equivalent. This method can be applied to samples with concentrations between 40 to 400 mgL^{-1} COD. Hence, the feed samples of > 400 mgL^{-1} COD required dilution with distilled water. In this study, all samples were filtered through a glass fibre filter paper (Whatman® GF/A grade) to remove suspended material before the test was carried out. The method is detailed in Standard Method 5220-C (APHA, 1998) and the standard deviation was within ± 5%.

4.6.2 TSS, MLSS, VSS

The Total Suspended Solids (TSS) and Mixed Liquor Suspended Solids (MLSS) were performed following the procedures presented in Standard Method 2540-D (APHA 1998). In order to determine the volatile fraction of the suspended solids concentration (VSS) an additional procedure was carried out following the total suspended solids determinations. This additional step included ignition of the filter papers at a temperature of 550°C and the determination of the fixed fraction of the solids. The volatile fraction of the solids was then calculated using the difference between the total solids and the fixed part of the solids as indicated in Standard Method 2540-E (APHA 1998). The coefficient of variation of 5 identical samples was within ± 5%.

4.6.3 Organic Carbon

This parameter was used to precisely measure and control the influent and effluent carbon concentrations, as it was particularly important to calculate the mass loading to the system and to observe the treatment efficiency. Samples were first filtered using a glass fibre filter paper (Whatman® GF/A grade) having a pore size ranging between 1.2 and 1.6 μm. The analysis was then performed by a SHIMADZU-5050A Total Organic Carbon Analyser (Shimadzu Corporation, Kyoto, Japan). The analszer yielded the value of Total Carbon (TC) and Inorganic Carbon (IC) for each sample separately. The TOC value was then automatically calculated by using the analyser from the difference (TOC = TC – IC) of the readings. The coefficient of variation of 5 identical samples was within ± 2%.

4.6.4 pH

Throughout the study, the pH values of the feed, mixed liquor in reactor and the reactor effluent were measured using a JENWAY 3310 pH meter (Jenway Limited, Essex, UK). The pH probe was calibrated daily with standard buffers prior to use and values obtained were accurate to within ±0.02 units.

4.6.5 Biogas Composition

The system performance was evaluated by methane yield. For determination of composition of the biogas, a sample was collected using a 1 mL glass syringe which was analysed by gas chromatography (Becker model 403 Gas Chromatography with Unicam 4815 integrator) under operating conditions as

follows: column temperature of 55°C, column dimension of 2000 mm long x 4 mm I.D. packed with Porapak Q, detector thermal conductivity, carrier gas: helium at 50 $mL.min^{-1}$. Each gas composition produced was multiplied for the calculation of percentage composition with the following constants (obtained from monthly calibration): CO_2 1.00; CH_4 1.328; residual air (or N_2 in the absence of gas-leaks) 0.744. The coefficient of variation of 5 identical samples was within ± 2%.

4.6.6 Biogas Production Rate

Gas production was monitored daily during each phase using an optical gas-bubble counter (Chelliapan, 2006) having a measurement range of 0 – 1.5 $L.h^{-1}$ and precision within ±1%. The operation principle involved biogas bubbles tripping a optical sensor (counter) and each bubble volume being calibrated previously by passing a known volume of gas through the device (fortnightly).

Table 4.3: Analysis & Sampling Schedule.

Anaerobic Membrane Bioreactor (AMBr) Phase I, II, III & IV		
Parameter	**Test Frequency**	**Sampling Location**
Gas Composition	daily	Gas line
Gas Production	daily	Gas meter
COD Feed	3 days/week	Feed tank
COD Reactor	3 days/week	Bottom sampling point
COD Permeate	3 days/week	Permeate line
pH: Feed	daily	Feed tank
pH: Reactor	daily	Bottom sampling point
pH: Permeate	daily	Permeate line

VFA: Reactor VFA: Permeate	3 days/week 3 days/week	Bottom sampling point Permeate line
Nitrogen & phosphate: Feed, Reactor and Permeate	monthly	Feed tank, bottom sampling point and permeate line
Nitrate & sulphate: Feed Nitrate & sulphate: Reactor Nitrate & sulphate: Permeate	weekly weekly weekly	Feed tank Bottom sampling point Permeate line
SS/VSS: Reactor SS/VSS: Permeate	weekly weekly	Bottom sampling point Permeate line
MCPP concentration: Feed MCPP concentration: Reactor MCPP concentration: Permeate	weekly weekly weekly	Feed tank Bottom sampling point Permeate line
Bacterial population (FISH)	every different condition (only Phase I,II,III & IV)	Bottom sampling point

4.6.7 Volatile Fatty Acids

Samples from reactor and permeate lines were first filtered using a glass fibre filter paper (Whatman® GF/A grade) having a pore size ranging between 1.2 and 1.6 μm to protect column. The ATI UNICAM 610 Series gas chromatograph with auto-injector and PU 4811 computing integrator operates under the following conditions: carrier gas: nitrogen at 20 ml/min; column temperature 140°C; detector type & temperature: flame ionisation detector (FID) & 180°C, column dimensions: 2000 mm long x 2 mm I.D. glass packed with 10% AT-1000 on 80/100 Chromosorb W-AW. The coefficient of variation of 5 identical samples was within ± 2%.

4.6.8 Ion Chromatography

Presence of nitrate (NO_3^-) and sulphate (SO_4^{2-}) in the permeate was monitored in Phase II and III to verify the denitrification and sulphate-reducing processes were well functioning. Sample filtration procedure was initially carried out as mentioned in Section 4.6.3 to protect the column of the Dionex, ICS-1000 Ion Chromatograph fitted with AS40 Automated Sampler, and data analysis carried out by Chromeleon software (Dionex, Corporation). The coefficient of variation of 5 identical samples was within ± 2%.

4.6.9 Sludge Sampling and Fixation

At the end of each from Phase I, II, III and IV, sludge samples were taken from the bottom of AMBr sampling port and transferred into sterile 20 ml graduated Universal bottles and fixed with absolute ethanol in a 1:1 ratio (1 volume ethanol: 1 volume sample) (Manz *et al.*, 1994) for total bacterial count with the fluorechrome (fluoresecent stain), 4', 6-diamidino-2-phyenlindole (DAPI, Sigma, Doroct, UK).

For fluorescent *in situ* hybridisation (FISH), the sludge samples taken from the bottom of AMBr sampling port were then fixed with 4% paraformaldehyde and the following steps performed for cell fixation. The samples (2 mL) were washed with 2 ml Phosphate Buffer Saline (PBS) by vortexing for a few seconds. The sample was centrifuged for 3 minutes at 13,000 rpm. Then this step was repeated again by pouring supernatant and adding 2 ml PBS. Subsequently, the supernatant was decanted and 0.5 ml PBS was added to the pellet that was resuspended by vortexing.

Afterwards 1.5 ml cold fixative containing 4% paraformaldehyde was added to the cell suspension. The sample was then left to incubate at 4°C overnight. Fixed cells were washed by centrifuging at 13,000 rpm (Hermle, Z160M) for 3 minutes, removing the supernatant and adding 2 ml PBS and vortexing. Finally, the cells were centrifuged again at 13,000 rpm for 3 minutes. The supernatant was removed and PBS: absolute ethanol (1:1, v/v) added to give a total volume of 2 ml. If required this suspension was stored long-term at -20°C (6 months to a year). However, prolonged storage in fixative is thought to reduce the quality and strength of the fluorescent signal (Amann *et al.*, 1990).

4.6.10 Total Cell Counts (TCC)

Total bacterial counts were made on reactor sludge (Phase I, II, III and IV) using the epifluorescent microscopy method described by Kepner and Pratt (1994) involving membrane filtration and DAPI staining. These counts were performed with the fluorechrome (fluoresecent stain), 4', 6-diamidino-2-phyenlindole (DAPI, Sigma, Doroct, UK) which stains all nucleic acids by binding directly to polyphosphates. Stock DAPI solution was stored in the dark at 4 °C. Different volumes of samples or different dilution series can be applied to obtain a suitable bacterial density in the microscope field of view. In this study, three dilutions were made for each sample; 1/10, 1/100 and 1/1000 in order to obtain counts averaging between 30 and 300 cell per field.

100 µl DAPI stock (0.033 $\mu g.\mu l^{-1}$) was added to the Eppendorf tubes containing 10 µl samples and the total volume was made up to 1000 µl with MiliQ water. Eppendorf tubes were then vortexed and incubated for 30 minutes at room

temperature in the dark to allow time for the stain to react. Nucleopore black polycarbonate filters (0.1 μm) were used to filter the DAPI stained solution using a sterile Milipore stainless steel filter unit. 30 μl of the DAPI stained solution was added to the filter together with 70 μl of MiliQ water. The filter was swirled around for a few seconds and the vacuum pump applied.

After filtration, the filter was removed and placed on top of a single drop of Citifluor (Citifluor Ltd, Canterbury, UK) anti-fadent previously added to a clean microscope slide. After allowing the surface of the filter to air-dry for a few minutes, another drop of Citifluor was added on top of the filter and covered with a cover-slip. The slide was viewed by epifluorescent microscopy and cells appearing a bright blue colour were counted in 20 fields of view at a magnification of x 100. Total cell counts were calculated according to the following formula (Kepner and Pratt, 1994):

$$\text{Total number of cells per ml} = \frac{\text{Mean (or median) number of cells per FOV} \times \text{Total area of filter (mm}^2\text{)}}{\text{Area of FOV (mm}^2\text{)} \times \text{Volume of sample applied (0.03 ml)} \times \text{Dilutions}}$$

4.6.11 Procedure for fluorescent *in situ* hybridisation (FISH)

A range of 16S rRNA oligonucleotide probes were used and the oligonucleotide probes and their target groups are listed in Table 4.4. All hybridisations were carried out in solution after fixation and permeabilisation procedures (4.5.10). The cells which were stored in ethanol: PBS (1:1 v/v) previously were taken

(100-200 µl) and centrifuged for 3 minutes at 13,000 x g (Z160M, Hermle). After the supernatant was removed, the sample was serially dehydrated in successive steps of increasing concentrations of ethanol (60, 80, 96% v/v) for 3 minutes each and separated by centrifugation of 13,000 x g each.

Table 4.4: Oligonucleotide probes used for whole-cell hybridization.

Probes	Fluorophore	Specificity (rRNA target, position)	References
EUB338I	FITC	Bacteria (16S, 338-355)	Amann et al., 1990
EUB338II	FITC	Bacteria (16S, 338-355)	Daims et al., 1999
EUB338III	FITC	Bacteria (16S, 338-355)	Daims et al., 1999
ARC915	CY3	Archae (16S, 915-934)	Amann et al., 1990
MX825	CY5	Methanosaeta (16S, 821-844)	Rocheleau et al., 1999
MS821	CY5	Methanosarcina (16S, 821-844)	Rocheleau et al., 1999

The supernatant was removed in each step and suspension was mixed as the ethanol was added. 2 µl of probe (50 ng.µl^{-1}) was added to the sample and incubated at the optimal hybridisation temperature of 46 °C overnight. The hybridisation buffer was added so that the final volume including the probe was 40 µl. However, for the negative control for autofluorescence (containing no probe), 40 µl HB was added. For a hybridisation containing only one probe (2 µl), 38 µl HB (Manz *et al.*, 1992) was added and for a hybridisation containing two probes (dual hybridisation; 2 + 2 µl) 36 µl HB was added.

After hybridisation, the sample was centrifuged at 13,000 *g* for 3 minutes and the supernatant was removed. A 0.5 ml of wash buffer was added to the resultant

pellet and mixed using a pipette and incubated for 15 minutes at 48 °C. These procedures of washing and incubation were repeated twice before washing the sample by adding 1 ml of filtered MiliQ water and centrifugation at 13,000 g for 3 minutes. The supernatant was decanted in all the above steps. Finally, the sample was resuspended with 100 µl of MilliQ water. A 10 µl aliquot of the sample is added to a Teflon-imprinted gelatine-coated slide (previously prepared). The sample was allowed to dry and the sample spot mounted on the slide in a single drop of the anti-fadent - Citifluor (AF1, Canterbury, UK). For viewing, a cover glass was placed over the preparation and the sample was viewed using an epifluorescence microscope or confocal scanning laser microscope (CLSM).

4.6.12 Hybridisation Buffer

1 ml stock hybridisation solution containing 20% formamide was prepared by mixing 0.2 ml of 4.5 M NaCl, 0.1 ml of 200 mM Tris-HCl (pH 7.2), 0.5 ml of MiliQ water, 10 µl of 10% SDS (Sodium Dodecyl Sulphate) and 0.2 ml of deionized formamide. This hybridisation buffer was adequate for about 25 hybridisations.

4.6.13 Washing Buffer

20 ml stock solution with 20% formamide was prepared by mixing 2 ml of 200 mM Tris-HCl (pH 7.2), 0.2 ml of 10% SDS, 1 µl of 4.5 M NaCl, 0.2 ml of 0.5 M EDTA (pH 8.0) and made up to volume by adding MiliQ water. Final ion concentrations in the washing buffer solution were 20 mM Tris-HCl, 0.1% SDS,

225 mM NaCl, 180 mM EDTA (Manz *et al.*, 1992). This washing buffer was adequate for about 20 hybridisations.

4.6.14 Preparation of Gelatine-Coated Slides

50 g KOH was dissolved in 500 ml of 95% ethanol and placed in a plastic container. A rack of approximately thirty slides was immersed in this solution for one hour. The rack of slides was removed and ethanol was replaced with distilled water. The slides were then immersed in distilled water for thirty seconds and shaken. Then, the distilled water was replaced and these two washing steps were repeated three times. After the final wash, the rack with slides was left to air dry. A mixture of 0.5 g gelatine and 0.05 g Chrome alum $CrK(SO_4)_2$ was dissolved in 500 ml of hot distilled water heated to 70 °C in a plastic microscope slide box placed in a water bath. The dry rack of slides was immersed for three minutes in this coating solution and then removed and allowed to air-dry for 5 minutes. This washing step was repeated three times. Then the rack of slides was left overnight to dry fully in a dust free place. The prepared slides were stored in an air-tight container placed in the dark at 4 °C. Multispot microscope slides (eight 5 mm diameter wells) (C.A. Hendley – Essex Ltd) were used in the FISH analysis (Section 4.6.11).

4.6.15 Confocal Laser Scanning Microscope (CLSM)

The slides were viewed using a Confocal Laser Scanning Microscope (CLSM) (Model Leica TCS SP2 UV–DMXRA) with magnification, X 63 1.32 Na lens (oil immersion). The associated software used was the Leica Confocal Software

Version 2.5, Build 1347 (Leica Microsystems, Germany) and all images were collected sequentially frame by frame.

4.7 STATISTICAL ANALYSIS

4.7.1 AMBr Performance

Statistical analysis of data was carried out in order to confirm whether differences existed between different conditions in each phase of the AMBr. Data analysis was performed by MINITAB V 14 (Minitab Inc., Phiadelphia, USA) using analysis of variance (ANOVA) to compare mean values. Mean values of COD removal and methane yield for each concentration of (*RS*)-MCPP (Phase I), each COD/NO_3^{-1}-N ratio (Phase II), each COD/SO_4^{-2} ratio (Phase III), and each HRT (Phase IV & V) were based on the mean of the final four consecutive points taken when the reactor had approached 'steady-state'.

4.7.2 Bacterial Population of AMBr Sludge

Cell counting was carried out according to the statistical method proposed by Davenport and Curtis, 2004, which proposed the following procedures when analysing cells:

i. Dispersion ratio (varience/mean) is calculated for each of the counts;

ii. Checking the data for normality and homogeneity of variances;

iii. Nested ANOVA to be used for determining the level of the greatest variation;

iv. Determination of the sample size;

v. Significance test, e.g. *t*-test or ANOVA.

Twenty random observations were used to determine the number of cells per field of view (FOV) for DAPI-stained. The variance within these levels was determined using nested analysis of variance with the MINITAB V14 program (Minitab Inc., Philadeiphia, USA). An example analysis is given in Appendix II.

4.8 HPLC ANALYSIS OF (*RS*)-MCPP

4.8.1 Extraction Procedure for (*RS*)-MCPP

Sample preparation is a vital part of HPLC analysis, in order to provide a reproducible and homogeneous solution that is suitable for injection onto column. In this study, solid phase extraction (SPE) was used for sample pre-treatment prior to injecting the sample onto the HPLC. The objectives of sample preparation are:

i. To wash away impurities in samples;

ii. To protect the stationary phase of the column.

75 ml aqueous samples were filtered using a glass microfibre filter paper (Whatman® GF/A grade) having a pore size ranging between 1.2 and 1.6 µm and filtrate pH was adjusted to 5.5 to 7.5 using 1 M HCl. A 60 mg/3 mL SPE strata-X polymeric sorbent (Phenomenex) was conditioned using 3 ml methanol and equilibrated using 3 mL water. Following 75 mL sample application at 4 $mL.min^{-1}$ the column was washed using 1 mL water. Residues were eluted using 1.2 mL methanol. The volume of the eluent was evaporated to 0.2 mL using a gentle stream of N_2 gas and reconstituted to 1 mL with water.

4.8.2 Analyte Recovery in Extraction Procedure

Accuracy of the extraction method was estimated using the parameter of recovery and reproducibility. A replicate of six permeate samples were prepared and five of these were spiked with 20 mgL^{-1} of (*RS*)-MCPP standard each. The last sample acted as a control sample and these six samples were extracted as mentioned in Section 4.8.1 before injection onto HPLC to determine (*RS*)-MCPP concentration. The recovery of each spiked sample was determined by the difference in concentration of the spike and the control. The recoveries obtained were 89.05, 90.30, 94.25, 90.84 and 94.87% at a relative standard deviation (RSD) of 2.56%. The equation used to determine the percentage of recovery is shown below:

$$\text{Percentage recovery} = \frac{\text{Recovery } (mg.L^{-1})}{\text{Spiked value } (mg.L^{-1})} \times 100\%$$

Table 4.5: Analyte recovery in extraction procedure.

Sample	Concentration ($mg.L^{-1}$)	Recovery ($mg.L^{-1}$)	% Recovery
Control	37.60	-	-
Spiked 1	55.41	17.81	89.05
Spiked 2	55.66	18.06	90.30
Spiked 3	56.45	18.85	94.25
Spiked 4	55.77	18.17	90.84
Spiked 5	56.57	18.97	94.87

4.8.3 Development of Liquid Chromatographic Method for (*RS*)-MCPP

Several steps needed to be considered before beginning the development of a reliable protocol for (*RS*)-MCPP analysis in aqueous sample. One of the most important factors was the information concerning sample composition and properties such as molecular weights, chemical structures, solubility and concentration range of compounds in samples of interest (Snyder et.al., 1997). Chromatographic methods can be divided into two major categories: gas chromatography (GC) and high performance liquid chromatography (HPLC). HPLC is a separation technique useful for semi volatile and non-volatile chemicals, or for analytes that decompose upon heating (EPA, 1996) compared to GC which is more suited to volatile compounds. On the basis of this information, HPLC was selected on the basis of (*RS*)-MCPP being a non-volatile compound.

4.8.4 Selection of Analytical Column

The reverse-phase C18 column was chosen over C8 because of its ruggedness, highly retention and widely used (Snyder et.al., 1997). Initially two C18 columns with different dimensions, lengths and column particle sizes namely, Phenomenex Synergi POLAR-RP (4 µm, 150 x 4.60 mm) and Phenomenex Gemini (5 µm, 250 x 4.60 mm) were used to analyse (*RS*)-MCPP aqueous sample using the same operating condition (composition and type of mobile phase, flow rate, detector wavelength etc). These two C18 columns are silica-based which is the most popular column packing material compared to porous polymer and other inorganic support materials. Increases in column length will

increase (*RS)*-MCPP retention times, but this decreases with column particle size allowing faster separations and higher efficiency.

However, smaller particle size columns have a tendency to plug more easily and decrease the column lifetime. The resolution of (*RS)*-MCPP peak for each column was compared in terms of peak shape and retention time (Figure 4.5). Even though Phenomenex Synergi POLAR-RP gave shorter (*RS)*-MCPP retention time than the Phenomenex Gemini column (because of its shorter length dimension, 150 mm), nevertheless it had secondary retention (peak asymmetry) at the end of the peak which reduced precision of peak area analysis.

Table 4.6 shows that the (*RS)*-MCPP peak area using Phenomenex Synergi POLAR-RP is greater only by 1.18% compared to the Phenomenex Gemini column and this was due to asymmetrical peak. The secondary retention might be caused by silanol effects (Snyder et.al., 1997) which are caused by the interaction of samples with the silanols of silica-based column can lead to increased retention, peak tailing (asymmetrical) and column to column irreproducibility. According to the manufacturer's manual-sheet, Phenomenex Gemini has low silanol activity at pH 2.5 compared to Phenomenex Synergi POLAR-RP which could contribute to silanol effects. Furthermore, pH stability of Phenomenex Gemini is wider, 1-12 pH unit compared to Phenomenex Synergi POLAR-RP which is only in the range of 1.5 to 7.0 pH unit. In consideration of the asymmetrical peak shape, Phenomenex Gemini was selected against Phenomenex Synergi POLAR-RP for the analysis throughout the study.

Table 4.6: (*RS*)-MCPP peak comparison between Synergi and Gemini.

Analytical column	Retention time (min)	Area	Secondary Retention
Synergi	2.82	12,043,216	Yes
Gemini	5.80	11,900,802	No

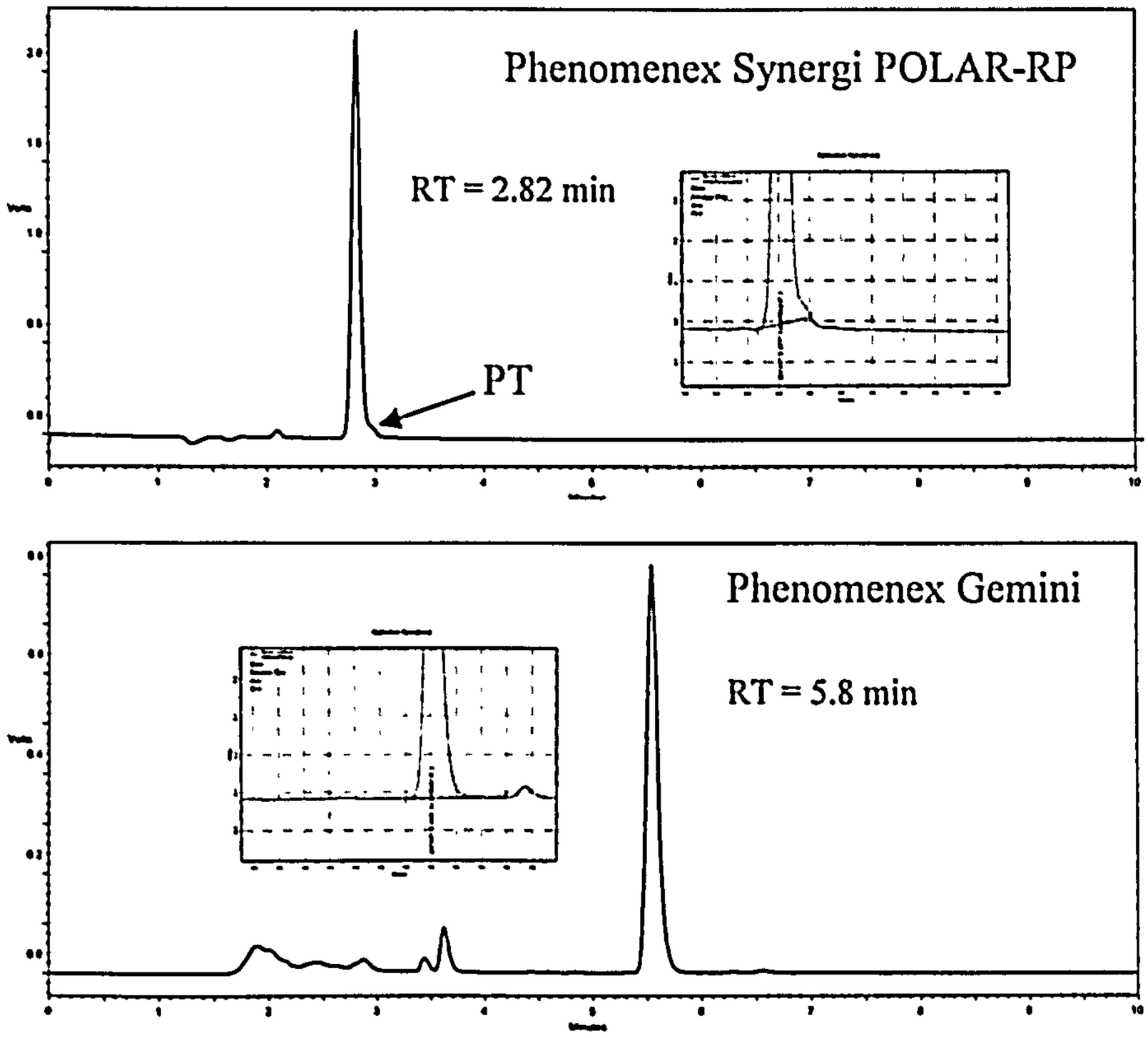


Figure 4.5: Comparison of (*RS*)-MCPP peaks under two analytical columns at (*RS*)-MCPP concentration of 10 mg.L^{-1} showing peak-tailing (PT).

4.8.5 Selection of Mobile Phase and Flow Rate

Initially, several different mobile phases were tested before selecting an acetonitrile and phosphate buffer. (*RS*)-MCPP retention can be controlled by varying mobile phase composition or solvent strength. A strong solvent decreases retention while a weak solvent increases retention. The method development was initially started using a composition of acetonitrile and water

(pH 2.50), acetonitrile and phosphate buffer (KH_2PO_4, 20mM), then methanol and phosphate buffer. Acetonitrile was the best initial choice of organic solvent for the mobile phase because it is compatible with UV detection at the short wavelengths and low UV intensities necessary for some saturated hydrocarbons substituted by ether, hydroxyl, chloro, carboxy or ester groups (Snyder et.al., 1997).

Furthermore, acetonitrile (0.38 cP) has a much lower viscosity than methanol (0.55 cP) resulting in lower column pressures which is desirable for column operation (Figure 4.6). Silanol effects can be minimized by using higher buffer concentration (> 10 mM) as the mobile phase, and buffers in the potassium form are more soluble in organic-water mobile phases than are buffers in the sodium form (Snyder et.al., 1997). Higher buffer concentration (> 50 mM) provides increased buffer capacity but may not be soluble in the mobile phase and furthermore may also adversely affect the operation of HPLC systems constructed from stainless steel. By comparing the chromatograms (Figure 4.6) it was discovered that the best resolution in terms of peak symmetry and elution time was achieved using acetonitrile and buffer (pH 2.50). According to Snyder et.al. (1997), an ideal flow rate for a column with an internal diameter of 4.6 mm will be 1.0 $ml.min^{-1}$, and it was decided to use the recommended flow rate without further testing.

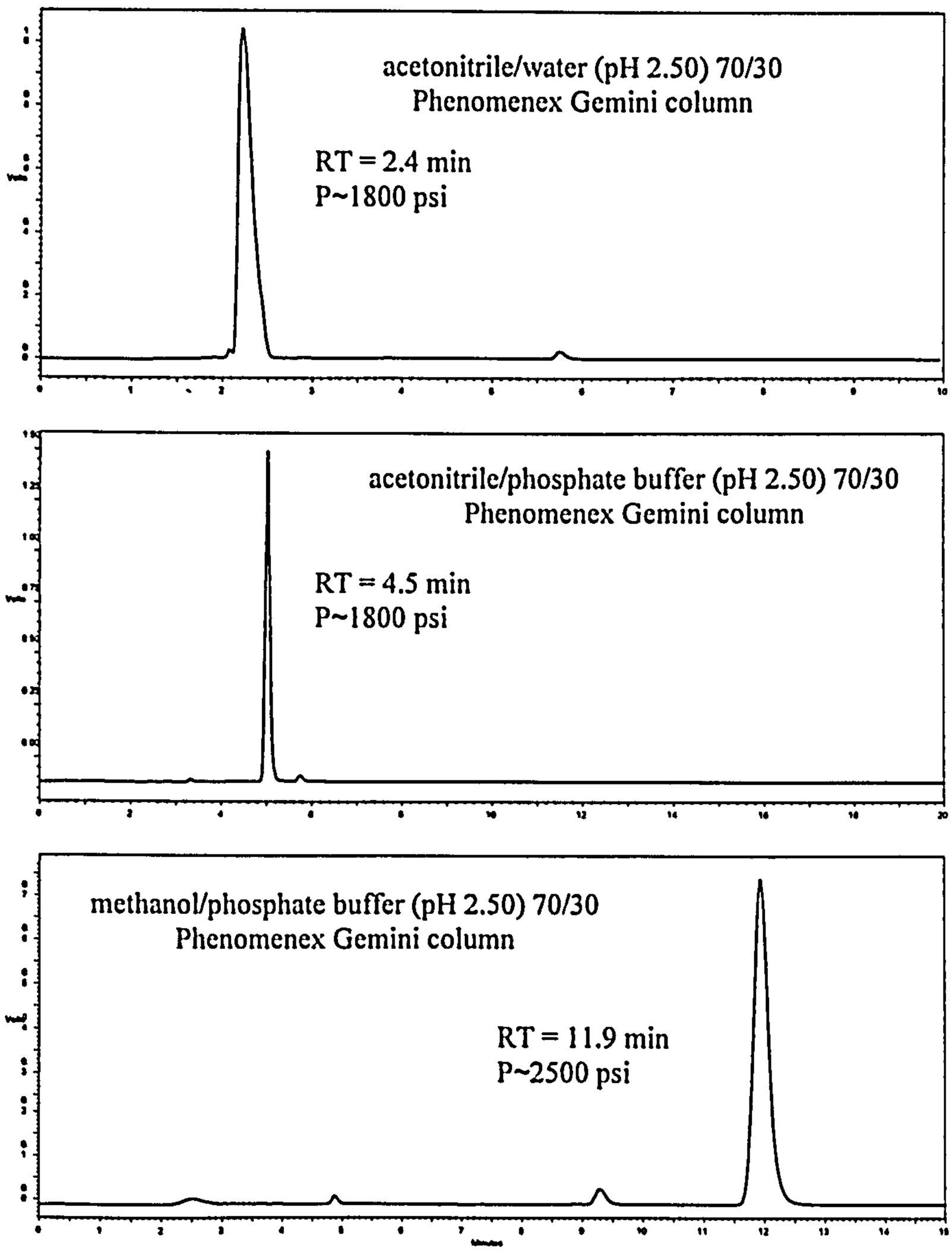


Figure 4.6: Comparison of (*RS*)-MCPP peaks under different mobile phases used at an (*RS*)-MCPP concentration of 40 mgL^{-1}.

4.8.6 Selection of Mobile Phase pH

pH optimisation is generally used to control band spacing for samples with more than one component, sample resolution and distorted peak. When optimising buffer mobile phase pH, it is useful to know the approximate pK_a values of the sample and the buffer itself, thus allowing mobile phase composition to be restricted to a useful range of pH values. It was important to adjust the mobile

phase pH accurately to the pKa value of (*RS)*-MCPP (pK_a=3.11) because when the mobile phase pH is close to the pK_a values, small changes as little as 0.1 unit in pH can have a major effect on (*RS)*-MCPP resolution.

The pK_a of the phosphate buffer (KH_2PO_4) was 2.10, and the upper pH limit allowed with this buffer is pH 3.1 (Snyder et.al., 1997) because the benefits of minimising silanol affects will be reduced significantly when the mobile phase pH is more than 3.1. According to Snyder et.al., (1997), a mobile phase with marginal buffer capacity will give less reproducible separation for compounds that are partially ionized at the pH of the mobile phase. Furthermore, retention time may change over a period of time and distorted peaks may occur. Based on all the information gathered, it was decided to use phosphate buffer at pH 2.5.

4.8.7 Selection of Wavelength of HPLC Detector

Choices of wavelength will influence the signal to noise ratio for a given concentration of a substance. In this current research, the appropriate wavelength for detection of (*RS)*-MCPP standard at a concentration of 1 mgL^{-1} was determined manually over the range of 220-300 nm using optimum conditions stated in Section 4.8.4, 4.8.5 and 4.8.6. Figure 4.7 shows that the peak area of (*RS)*-MCPP at wavelength 229 nm was increased 8 fold compared to that at 250 nm and above. However, when the detector wavelength decreased to 220 nm, the peak area decreased by a factor of 0.6, giving the optimum wavelength for (*RS)*-MCPP detection 229 nm. Figure 4.8 shows that at detector wavelength of 250 nm the baseline noise was apparently increased compared to wavelength 229 and

280 nm, and limited the detection sensitivity and quantification efficiency of the sample.

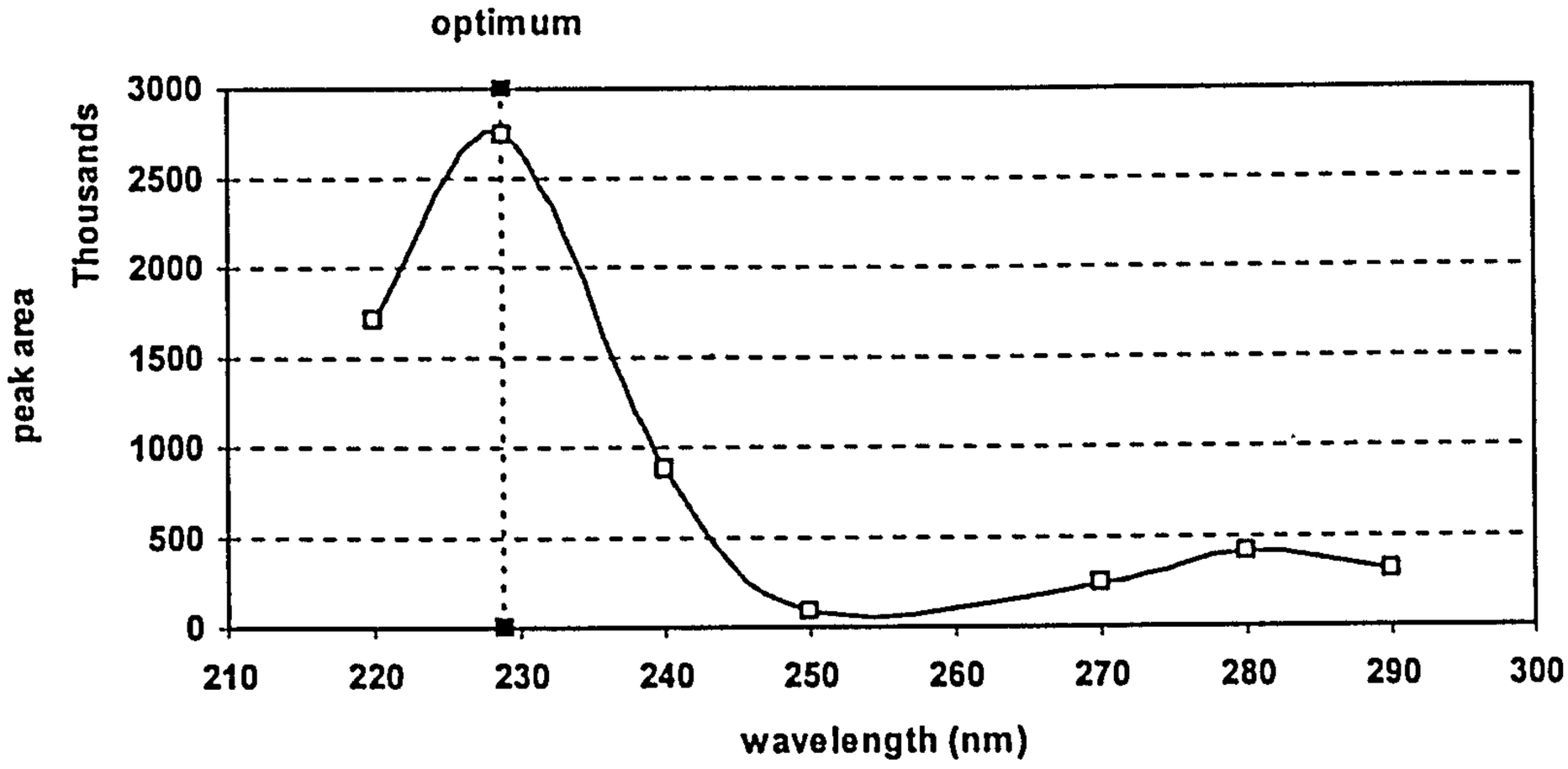
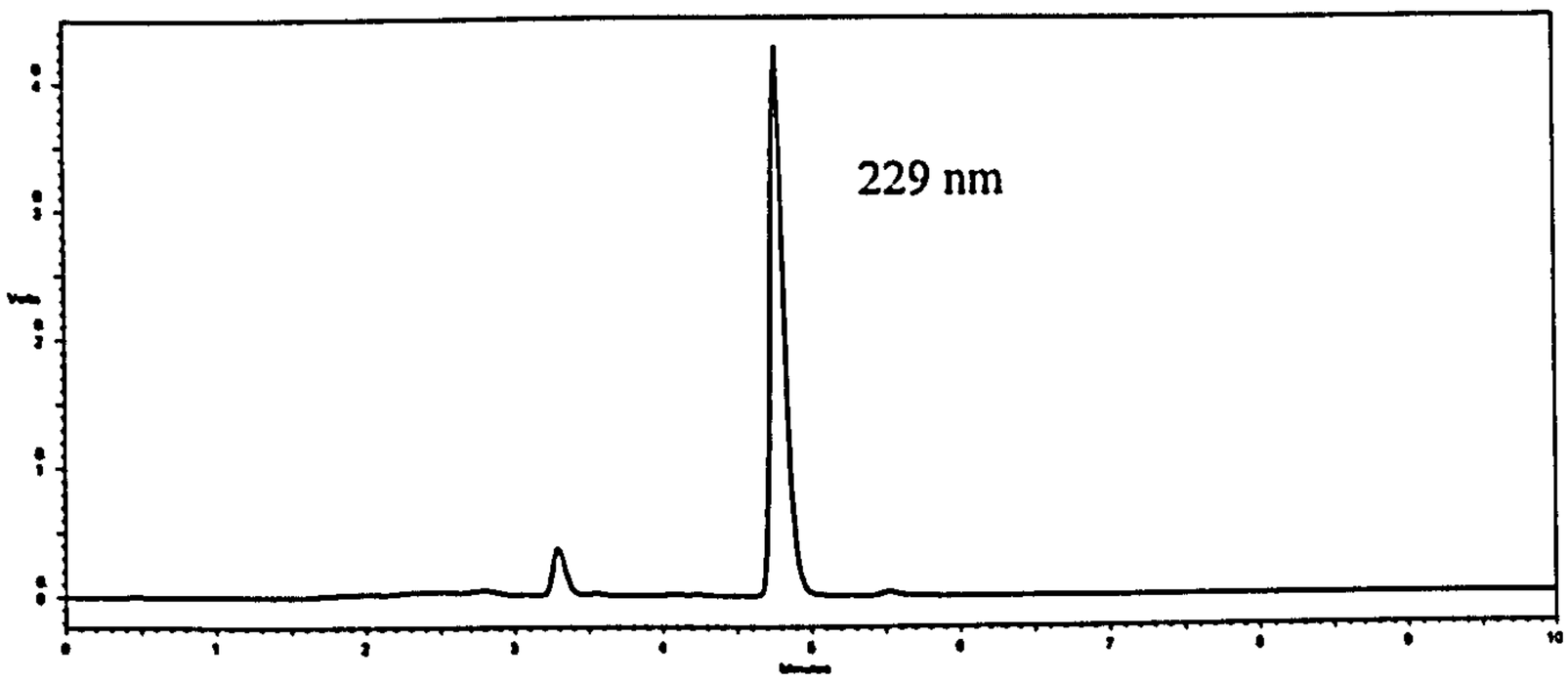
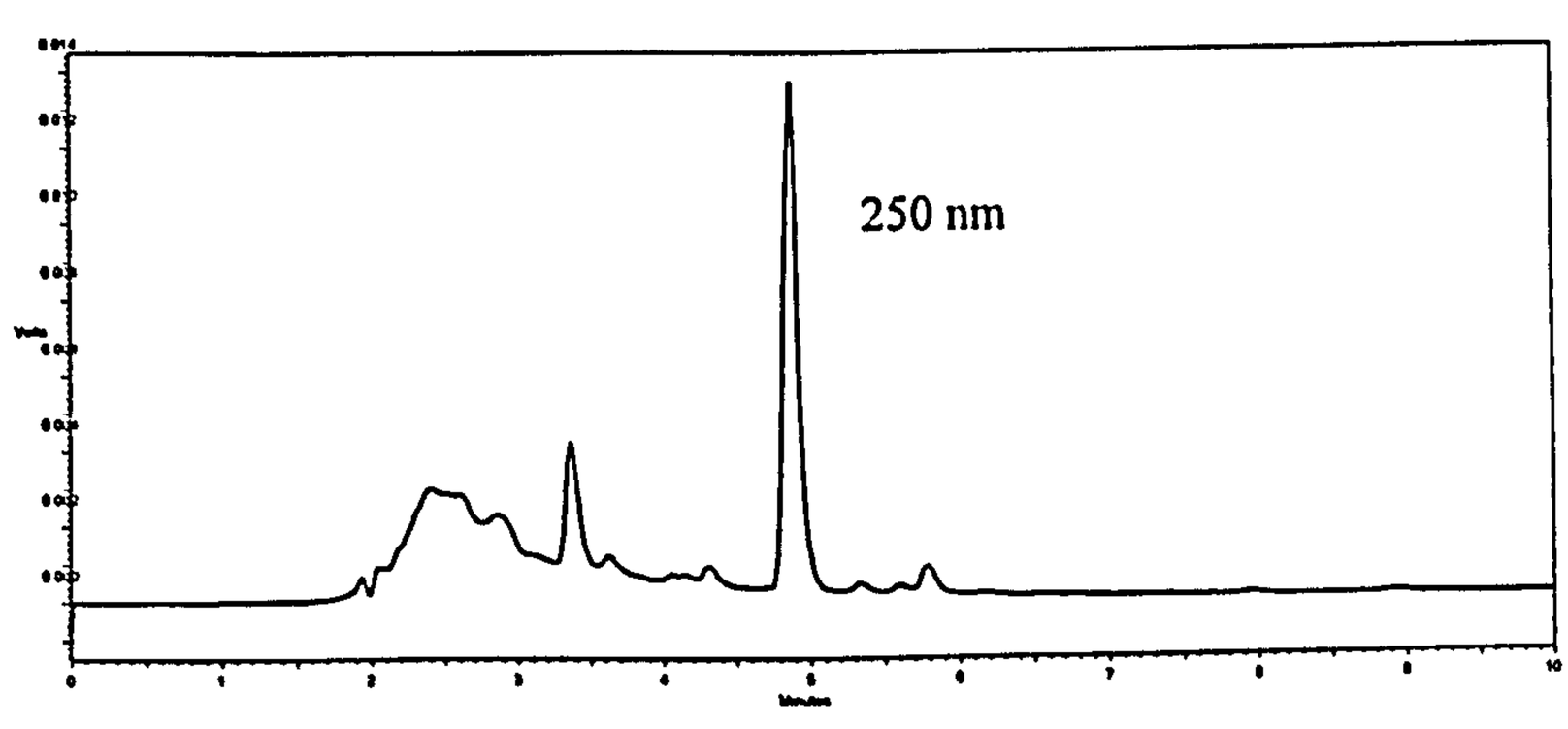


Figure 4.7: Peak area of *(RS)*-MCPP against different wavelengths.





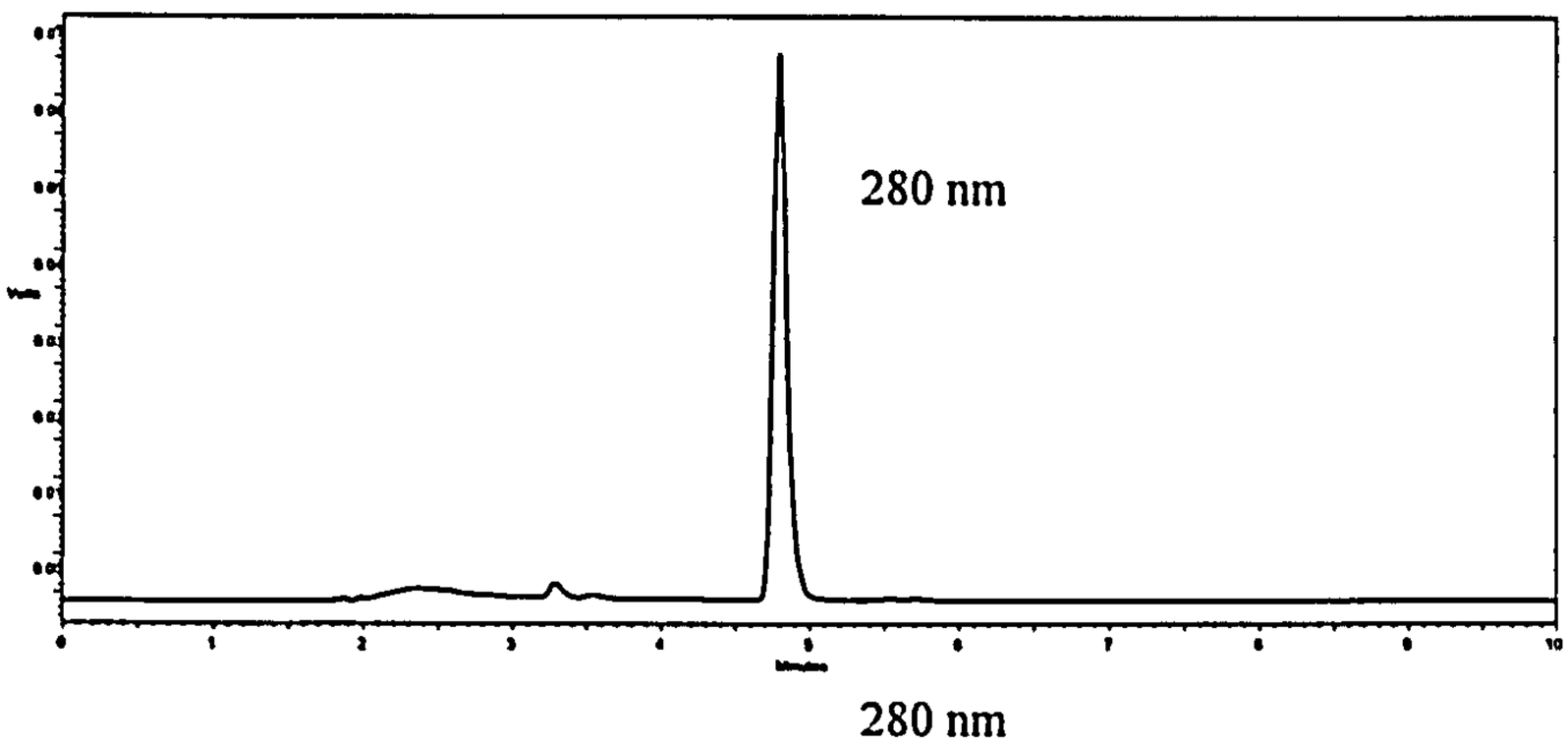


Figure 4.8: Comparison of *(RS)*-MCPP peaks under different wavelength used at *(RS)*-MCPP concentration of 1 mgL^{-1}.

4.9 OPTIMIZED HPLC CONDITIONS

Stationary Column:		Phenomenex Gemini C18 (5 µm, 250 mm L x 4.60 mm ID)
Guard Column	:	Polar-RP 4 mm L x 3.0 mm ID, Cat.No. AJO-6076
Mobile Phase	:	Acetonitrile HPLC Grade (BDH EC No.200-835-2)
		6±0.01 phosphate buffer (K_2HPO_4) g dissolved in water HPLC Grade (CAS 7732-18-15) adjusted to pH 2.5 using concentrated phosphoric acid (30 mM)
		(65:35, v/v)
Detection	:	UV-VIS ultraviolet visible light absorbance detector at 229nm
Flow Rate	:	1.0ml/min
Temperature	:	Room Temperature (~20°C)

Injection Volume : 10-20µl

4.10 METHODS OF QUANTITATIVE ANALYSIS

An external standard method was used to measure the concentration of both aqueous and biomass samples. Prior to the analysis of *(RS)*-MCPP from both aqueous and biomass samples, calibration curves (standard area of *(RS)*-MCPP vs. concentration in mgL^{-1}) were performed in duplicate by injecting five standard solutions of *(RS)*-MCPP (0.1 $mg.L^{-1}$, 1.0 $mg.L^{-1}$, 5 $mg.L^{-1}$, 10 $mg.L^{-1}$ and 20 $mg.L^{-1}$) onto HPLC. The relationship between the response of the HPLC and the standard concentration of *(RS)*-MCPP in mathematical equation was obtained and used to analyse sample concentration along this research.

CHAPTER FIVE

THE EFFECT OF ELEVATED (*RS*)-MCPP CONCENTRATIONS UNDER METHANOGENIC CONDITIONS

5.1 INTRODUCTION

There is very little information in the literature on the effect of (*RS*)-MCPP on biological treatment plants and any literature available is limited to investigating (*RS*)-MCPP degradation by aerobic systems (González et al 2006) using MBR. Furthermore there is no published information in the literature investigating the effect of elevated (*RS*)-MCPP by AMBr.

Although a low concentration of (*RS*)-MCPP has been detected in the environment in the range of nano- to micro-grams per litre (Bucheli et al., 1998; Petrovic & Larsson-Kovach, 1996; Zipper, et al., 1998; Fletcher et al., 2004), considerably higher concentrations might arise as a result of discarded batches, equipment failure and poorly functioning treatment plants at manufacturing sites (Section 1.1).

Therefore, the objectives of this chapter are two fold, firstly to investigate the effects of elevated (*RS*)-MCPP concentrations on AMBr reactor performance under methanogenic conditions and secondly to assess the efficiency of AMBr in degrading (*RS*)-MCPP under these conditions.

5.2 METHODS

The AMBr was seeded with anaerobic digested sewage sludge at Hexham municipal sewage treatment plant (Northumberland, UK) by the seeding procedure stated in Section 4.5, Chapter 4. The reactor was fed with synthetic (*RS)*-MCPP wastewater by adding the desired amount of primary solution of (*RS)*-MCPP to brewery wastewater. Technical and analytical grade (*RS*)-MCPP was a gift from AH Marks and Co. Ltd. (West Yorkshire), and was a racemic mixture of (*RS*)-MCPP with an isomer ratio of 1:1. (*RS)*-MCPP was added into the system on day 21 based on the initial assumption that a steady-state condition had been reached on that day based on COD removal efficiency data but data later showed that a steady state condition was reached approximately 10 days (day 31) after the initial assumption (Figure 5.2)

The concentration of (*RS)*-MCPP was increased stepwise throughout the experiment from 5 $mg.L^{-1}$ to 200 $mg.L^{-1}$ whilst operating under methanogenic conditions with an average OLR of 1.51(±0.20) kg COD $m^{-3}.d^{-1}$ at an HRT of 3.3 d (Table 5.1). The process performance of the reactor was characterised in terms of its pH, COD degradation, VFA accumulation, biogas production and composition, (*RS)*-MCPP degradation and MLSS & MLVSS.

Table 5.1: Summary of reactor operational conditions of the AMBr system during the study of elevated *(RS)*-MCPP concentrations.

OLR[a]	Day	HRT (d)	*(RS)*-MCPP ($mg.L^{-1}$)	Influent COD[b] ($mg.L^{-1}$)
1.51	1-20	3.3	0	5040
1.51	21-168	3.3	5	5040
1.51	169-206	3.3	20	5040
1.51	207-237	3.3	50	5040
1.51	238-262	3.3	200	5040

[a]= average value ($kgCOD.m^{-3}.d^{-1}$), [b]=average COD

5.3 RESULTS & DISCUSSION

5.3.1 (*RS*)-MCPP Stability Test

In order to determine the spontaneous degradation rate, a control sample of synthetic wastewater was analyzed for its (*RS*)-MCPP content on the day of preparation and was found to contain (*RS*)-MCPP at a concentration of 71.65 $mg.L^{-1}$. After 3 weeks incubation in a waterbath at 30°C without biomass, (*RS*)-MCPP concentration was 71.20 $mg.L^{-1}$ (0.6% difference), confirming that (*RS*)-MCPP was highly stable towards spontaneous abiotic degradation within the synthetic wastewater.

5.3.2 pH

The reactor was operated at an average OLR of 1.51(±0.20) kg $COD.m^{-3}.d^{-1}$ during start-up and the pH level increased rapidly from pH 6.5 to pH 7.5 then fluctuated soon after 5(±1) $mg.L^{-1}$ of (*RS*)-MCPP was introduced into the AMBr on day 21 (Figure 5.1). Initial fluctuations in both reactor and effluent (permeate) were attributed to acclimatization of the methanogenic bacteria to the (*RS*)-MCPP added but fluctuations soon attenuated (after 70 days) and pH then declined gradually from pH 7.4 to pH 6.6 (Figure 5.1).

Although there was no apparent increase in production of VFA (Figure 5.3), it is possible that formate was produced in increasing amounts during the course of the reactor run and was not converted fully to methane. These increasing levels formate would have led to a gradual decline in pH over time (as seen by the downward trend in pH seen in Fig 5.1). This cannot be confirmed because

formate was not detected by the flame ionization detector of the GC however indirect evidence to support the theory of increasing formate accumulation in the reactor exists as the reactor effluent did show gradual increase in COD over time (Fig 5.2).

Figure 5.1 also shows that the biomass response (pH fluctuation) immediately after starting each new concentration of (*RS*)-MCPP was less severe with each successive step, and proved an increasing level of acclimatization and stability of the biomass towards the (*RS*)-MCPP addition over time.

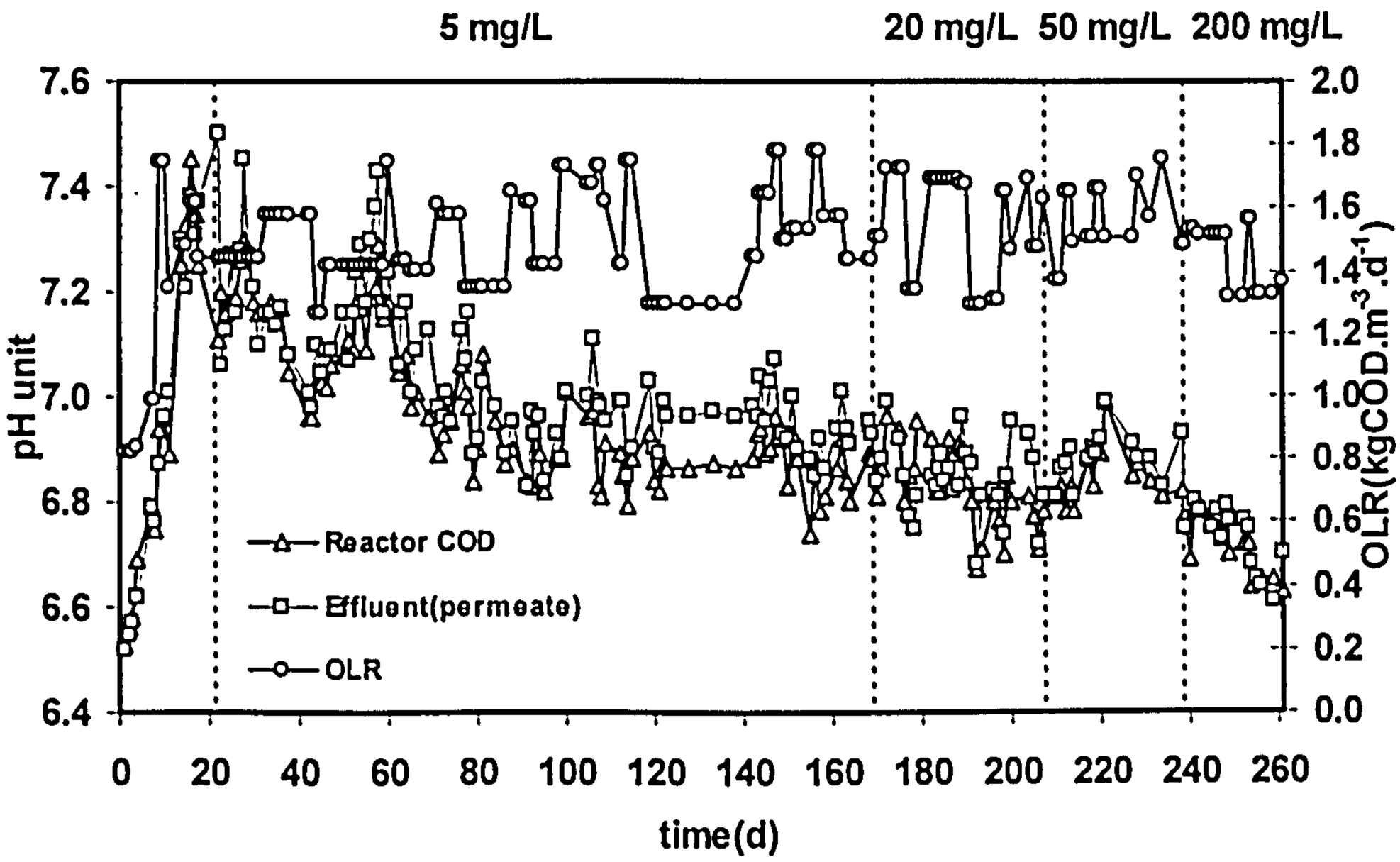


Figure 5.1: pH of the AMBr under different (*RS*)-MCPP feed concentrations.

5.3.3 COD Removal

The soluble COD removal efficiency in Phase I showed a generally stable profile and reached an average removal of 98(±0.7)% at *(RS)*-MCPP concentrations below 200(±3) $mg.L^{-1}$ (Figure 5.2), confirming the stability of the AMBr system towards *(RS)*-MCPP. However, at 200(±3) $mg.L^{-1}$ *(RS)*-MCPP the soluble COD removal efficiency decreased gradually over a period of 22 days to a value of 94(±1.5)% with a corresponding increase in effluent COD concentration from 120(±30) $mg.L^{-1}$ to 280(±66) $mg.L^{-1}$. Bacterial inhibition resulting from the high concentration of *(RS)*-MCPP (200(±3) $mg.L^{-1}$) probably caused the decreased removal efficiency of the soluble COD, however, at this concentration *(RS)*-MCPP contributed over 6.5% of the total influent COD, and the lower overall COD removal efficiency may have been due to the slower degradation kinetics of this organic substrate compared to those of the simpler carbon sources from the brewery wastewater.

Chemically, the structure of *(RS)*-MCPP is considered to be more recalcitrant than other members of the chlorophenoxyalkanoic acid family of herbicides, such as 2,4D or MCPA, since the propionic acid group is linked to the oxygen atom of the phenoxyacid moiety via the α-carbon atom adjacent to the carboxyl group (Kilpi, 1980) which imparts resistance to any enzyme breakdown. These results indicate that there were not any substantial inhibitory effects on the methanogens, even when *(RS)*-MCPP was present up to 50(±1) $mg.L^{-1}$ in the feed.

An increase in reactor COD after (*RS*)-MCPP addition to the system could be explained by the fact that the biomass produced microbial products (SMP) in response to environmental stress caused by the toxicity of (*RS*)-MCPP. SMP can be classified as utilisation-associated products (UAP) and biomass–associated products (BAP). UAPs are associated with substrate metabolism and biomass growth and are produced at a rate proportional to substrate utilisation which in this study is the methanogenesis rate. BAPs are associated with biomass decay and are produced at a rate proportional to biomass concentration (Barker & Stuckey, 1999). As the graph of reactor COD (Figure 5.2) is not linear, but shows a slight increase in gradient with time, SMP production rate could possibly be linked to the concentration of (*RS*)-MCPP, higher concentrations causing the bacteria to produce greater amounts of SMP.

Effluent COD would be largely unaffected by increasing SMP accumulation in the reactor due to the fact that the membrane would be likely to retain SMP in the reactor. Although it is not possible to confirm whether this was the case as there was no control reactor (without (*RS*)-MCPP) running in parallel during this experiment.

Another possible explanation might be the possibility that during the 260 days experiment there were significant changes in the biomass, which changed from a flocculant state to one containing greater numbers of free suspended cells (the latter would have passed through the GFC filter of the COD test thus increasing the COD values.

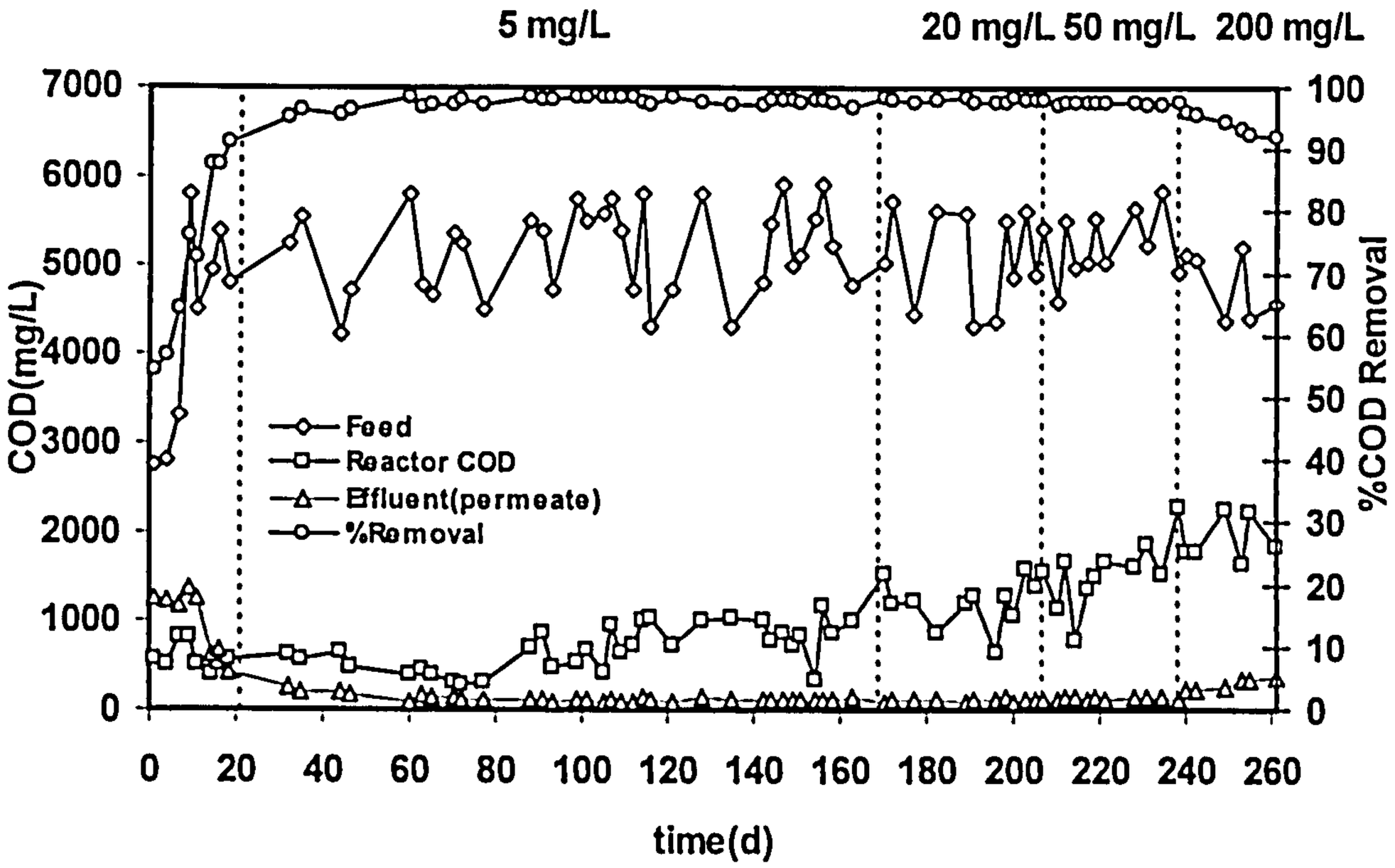


Figure 5.2: COD removal efficiency of the AMBr during Phase I under different (*RS*)-MCPP feed concentrations.

5.3.4 VFA

When 5 $mg.L^{-1}$ (*RS*)-MCPP was added on day 21, both acetic and propionic acid concentrations increased from 71.4 and 24.1 $mg.L^{-1}$ to 113.7 and 63.2 $mg.L^{-1}$ respectively on day 24. However, when (*RS*)-MCPP was increased to 20(±1) $mg.L^{-1}$, 50(±1) and 200(±3) $mg.L^{-1}$, the formation and degradation of acetate seemed to have little effect on (*RS*)-MCPP concentrations as acetate production was below 35 $mg.L^{-1}$.

Low acetate production could indicate that its production was reduced by the dominance of *Methanosaeta* which has a high affinity for acetate, $K_s = 20$ $mg.L^{-1}$

(Speece, 1996). This was further supported by a high composition of *Methanosaeta* species in the archael population described later in Chapter 9. These acetoclastic methanogens convert acetic acid to methane and bicarbonate. It has been estimated that from stoichiometric relations that about 70 % of the methane is produced via the acetate pathway (Hobson & Wheatley, 1993) which is in agreement with high methane production in the current study (Figure 5.4).

Equally, low propionate concentrations (after reaching steady-state) suggest low dissolved hydrogen concentrations in the AMBr. Although dissolved hydrogen concentrations were not measured in these experiments, it is generally accepted that propionate degrading bacteria, which are among the slowest growing members of the anaerobic consortia, depend on low hydrogen concentrations i.e. the partial pressure of hydrogen in the reactor should not be allowed to exceed 10^{-4} atm for efficient propionate degradation (Kasper & Wuhrmann, 1978). Hydrogen-producing acetogenic bacteria which are responsible for propionate degradation provide important substrates for methanogens such as acetic acid, H_2 and CO_2 and methanogens which in return act symbiotically removing H_2.

The formation and degradation of butyrate did not seem to be affected by the different (*RS*)-MCPP concentrations in the AMBr, as no significant concentrations of butyrate accumulated in the system at any time after start-up. McCarty & Mosey (1991) have proposed that butyrate is only produced in significant concentrations as a mechanism to counteract excessively low pH,

however in the current study pH was between 6.6 – 7.4 (Figure 5.1), well within the optimal range for anaerobic activity (Rittmann & McCarty,2001).

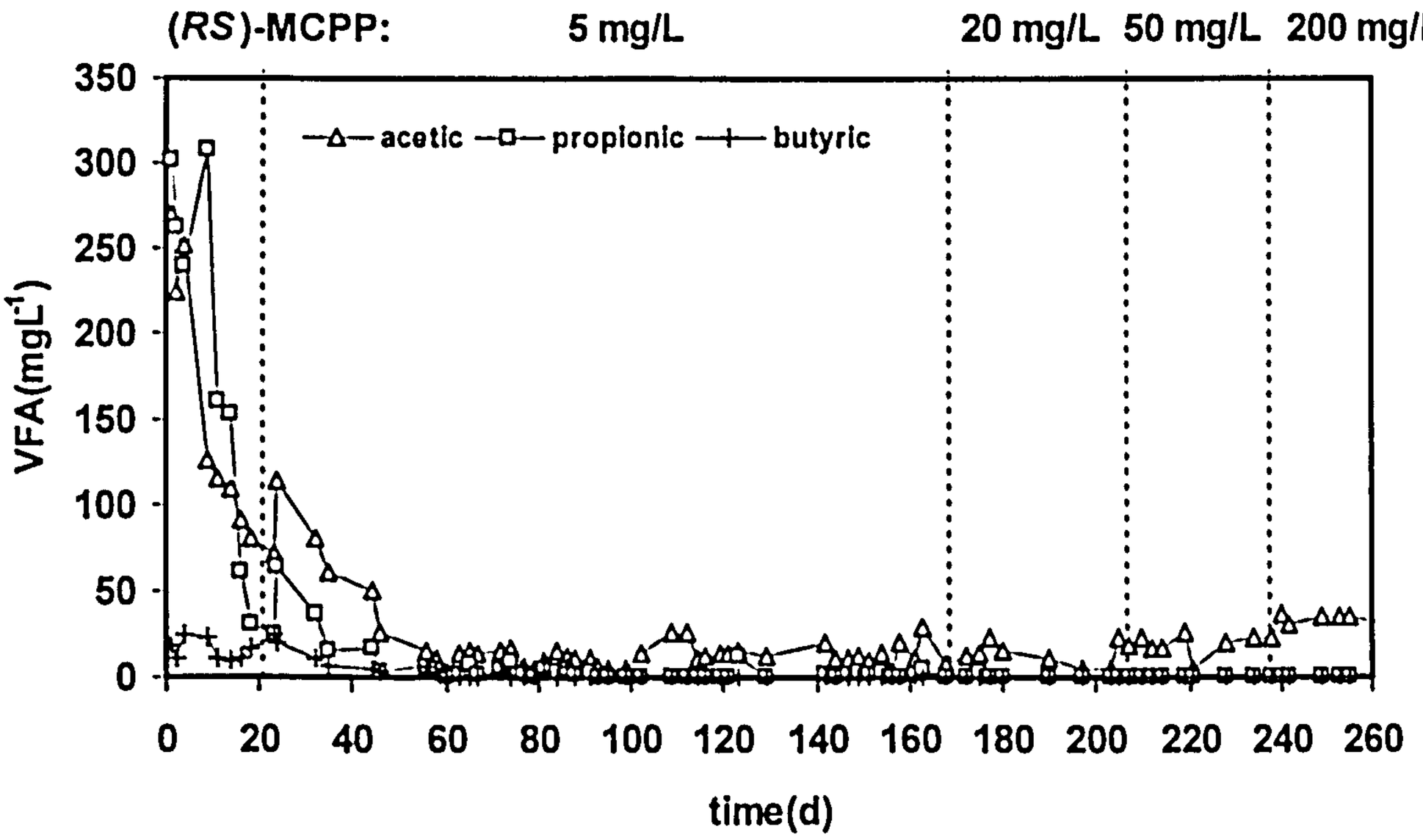


Figure 5.3: VFA concentration in the reactor during Phase I under different *(RS)*-MCPP feed concentrations.

5.3.5 Biogas

Although there appeared to be a severe decline in methane production between day 53 and 60 this was due to technical problems with the level controller which allowed water to enter the gas line (Figure 5.4). After day 21 when (*RS*)-MCPP was first introduced to the reactor (5(±1) $mg.L^{-1}$), the methane production rate dropped almost 12% from 5.9 $L.d^{-1}$ to 5.2 $L.d^{-1}$. However, this trend was not observed when feed concentration was increased to 20(±1) $mg.L^{-1}$ and 50(±1) $mg.L^{-1}$ of (*RS*)-MCPP due to biomass being acclimatized, a phenomenon also observed for data on both total VFA production and soluble COD removal efficiency. There was no severe stress shown by the methanogenic biomass

when 200(±3) mg.L^{-1} of (*RS*)-MCPP was added even though the methane production initially declined rapidly by 67% (7.35 L.d^{-1} to 2.4 L.d^{-1}) but soon recovered after 2 days.

In addition, data for the methane yield (values taken when the system approached steady-state) showed this parameter to be mainly unaffected by (*RS*)-MCPP concentration in the influent (Figure 5.6) because the theoretical value of methane conversion is 0.35 m^3 CH_4(STP)/kgCOD destroyed (McCarthy, 1964) or the experimental value 0.33 m^3 CH_4(STP)/kgCOD destroyed (Kennedy & van den Berg, 1982) neither of which are substantially different from the mean methane yield obtained in the current study (0.27-0.30 CH_4/kgCOD removed).

The average methane composition was high at 80(±2%) and this value is similar to that obtained by Chelliapan (2006) (between 75-80%) using same source of brewery wastewater, and as methane composition is dependent on the source and type of substrate (Speece & McCarty, 1964), proteins giving higher methane composition, it is likely that the waste beer used to prepare the reactor feed contained the substantial amounts of protein similar to those normally present in beer (Vanderhaegen, et al., 2006).

Biogas methane composition can also be affected by the reactor operating conditions, high pH favouring the dissolution (stripping) of carbon dioxide from the biogas leading to a greater proportion of methane being measured, and this

would almost certainly have contributed to the development of relatively high methane composition in the current study.

In contrast, the lower methane yield obtained in the early stage of the experiment (0.21 m^3CH_4/kgCOD destroyed) was due to the system having not reached steady state condition (cells in exponential phase of growth) causing carbon to be channelled into new biomass rather than methane.

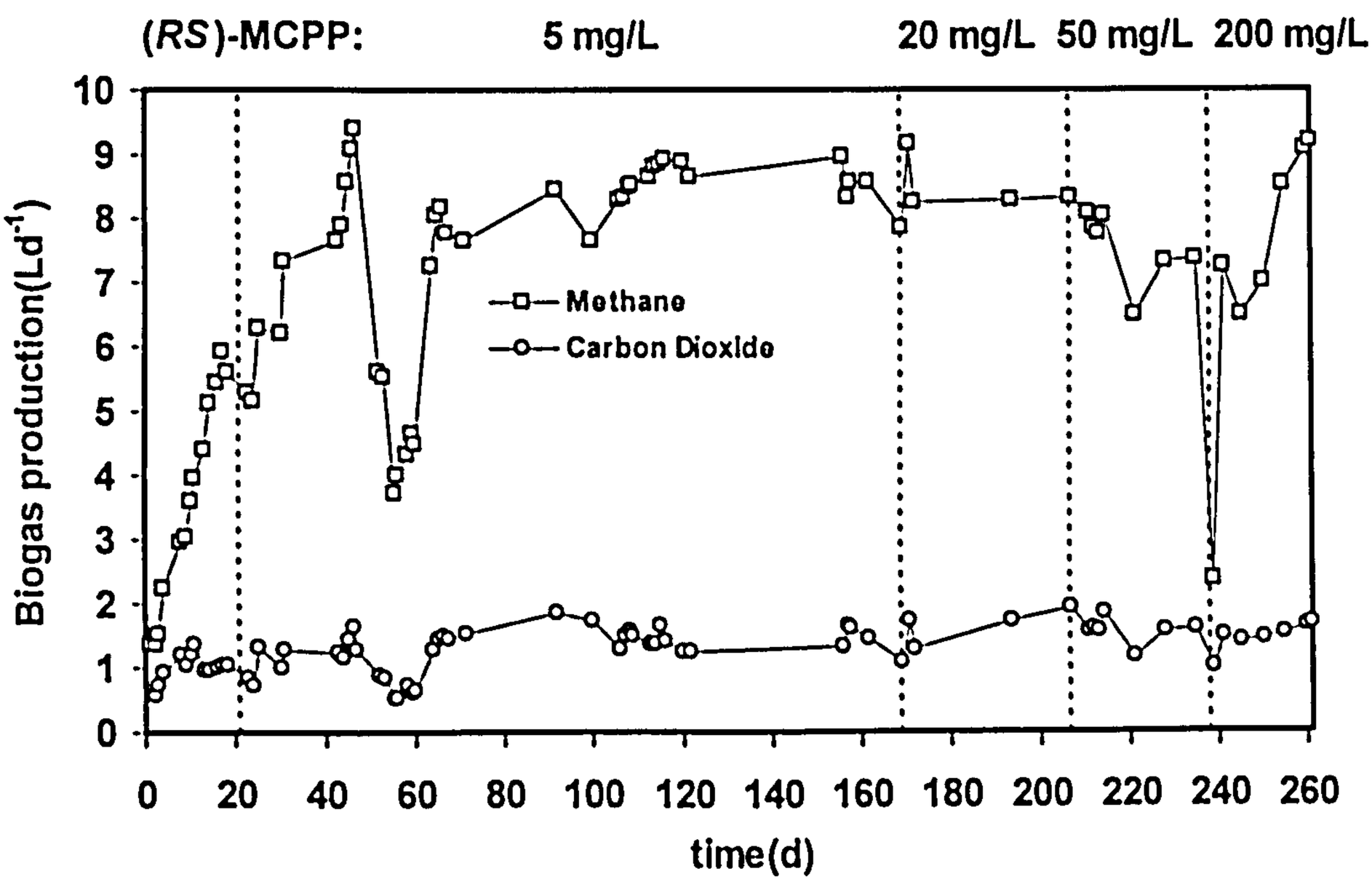


Figure 5.4: Biogas production in the AMBr during Phase I under different (*RS*)-MCPP feed concentrations.

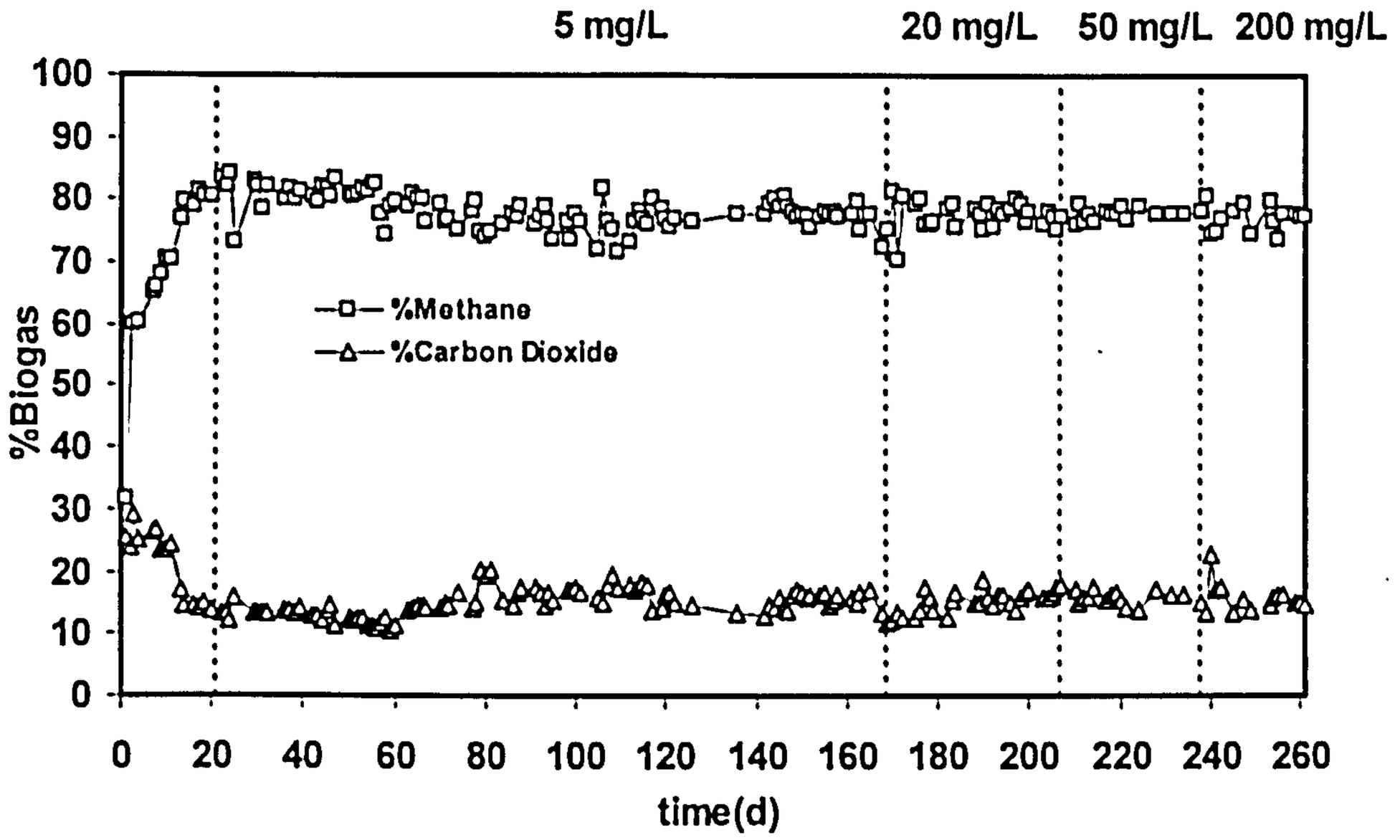


Figure 5.5: Biogas composition in the AMBr during Phase I under different (*RS*-MCPP) feed concentrations.

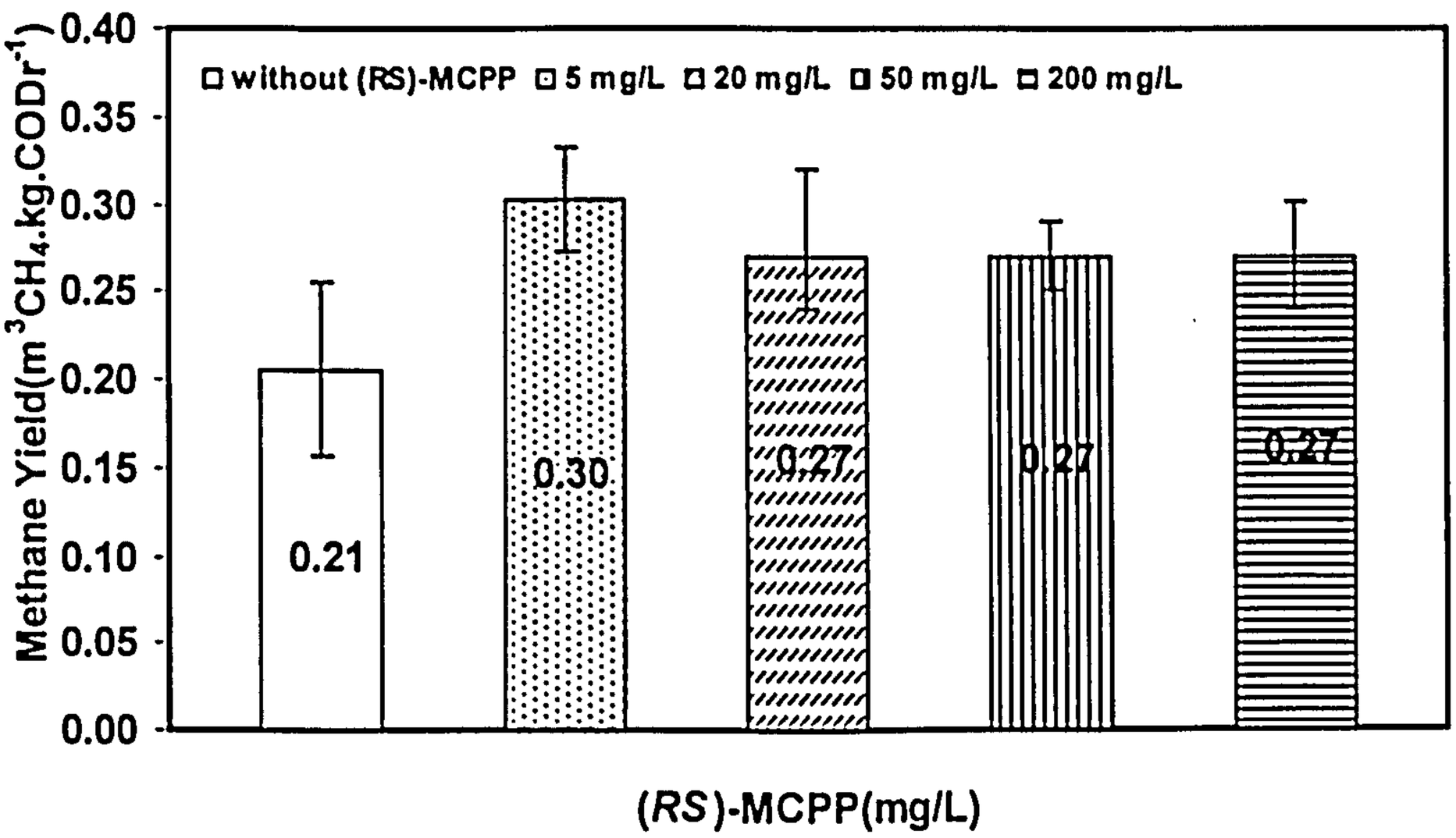


Figure 5.6: Mean methane yield in the AMBr during Phase I at different (*RS*)-MCPP concentrations (values taken when the system approached steady-state at each investigated (*RS*)-MCPP concentrations).

*data above taken from analysis on day 18 (without (*RS*)-MCPP), 158 (5mgL^{-1}), 203 (20mgL^{-1}), 234 (50mgL^{-1}), 261 (200mgL^{-1}).

5.3.6 (*RS*)-MCPP

In Phase I, the (*RS*)-MCPP concentration varied from 5(±1) $mg.L^{-1}$ to 200(±3) $mg.L^{-1}$ over a period of 261 days. At a dose of 5(±1) $mg.L^{-1}$ (*RS*)-MCPP, removal efficiency was around 20(±4) % but this decreased to less than 10% when 20-200 $mg.L^{-1}$ of (*RS*)-MCPP was applied. Even though the removal efficiency was low (Figure 5.7), the specific utilization rate of (*RS*)-MCPP increased from 0.15 (±0.02) $\mu g.mgVSS.d^{-1}$ (at (*RS*)-MCPP of 5 $mg.L^{-1}$) to 3.19(1.44) $\mu g.mgVSS.d^{-1}$ ((*RS*)-MCPP of 200 $mg.L^{-1}$ - Figure 5.8). Effects of (*RS*)-MCPP specific utilisation rates were more apparent at higher (*RS*)-MCPP concentrations.

In contrast, the COD specific utilisation rate was relatively less dependent on the (*RS*)-MCPP concentrations. There are two possible factors that might play an important role in determining the (*RS*)-MCPP SUR in the current study: acclimatization and concentration of (*RS*)-MCPP. Long acclimatization (170 days) is likely to increase the utilisation rate and this view is supported by Torang et al (2003). In their study on aquifer samples, they found that previous exposure of the aquifer to (*RS*)-MCPP resulted in reduced or no lag time before the onset of rapid aerobic degradation. This was further supported by Tuxen et al (2003) in a laboratory experiment which demonstrated (*RS*)-MCPP degradation occurred only after a long lag phase of 120 days in anaerobic microcosms using samples from an anaerobic landfill zone.

Specific studies of the effect of concentration in aerobic aquifers by Torang et al (2003) found that the biodegradation rate of (*RS*)-MCPP accelerated gradually at concentrations above 10 $\mu g.L^{-1}$, but not below this value.

Another interesting observation in the current study is the fact that the specific utilization rate for COD dropped at a (*RS*)-MCPP concentration of 200 $mg.L^{-1}$ wheras the (*RS*)-MCPP specific utilization rate improved with increasing (*RS*)-MCPP concentration up to and including 200$mg.L^{-1}$. The reason for this is not clear from the current study, and further study of the substrate utilisation rates of the individual (*RS*)-MCPP degrading and COD-degrading bacteria are needed to explain this observation.

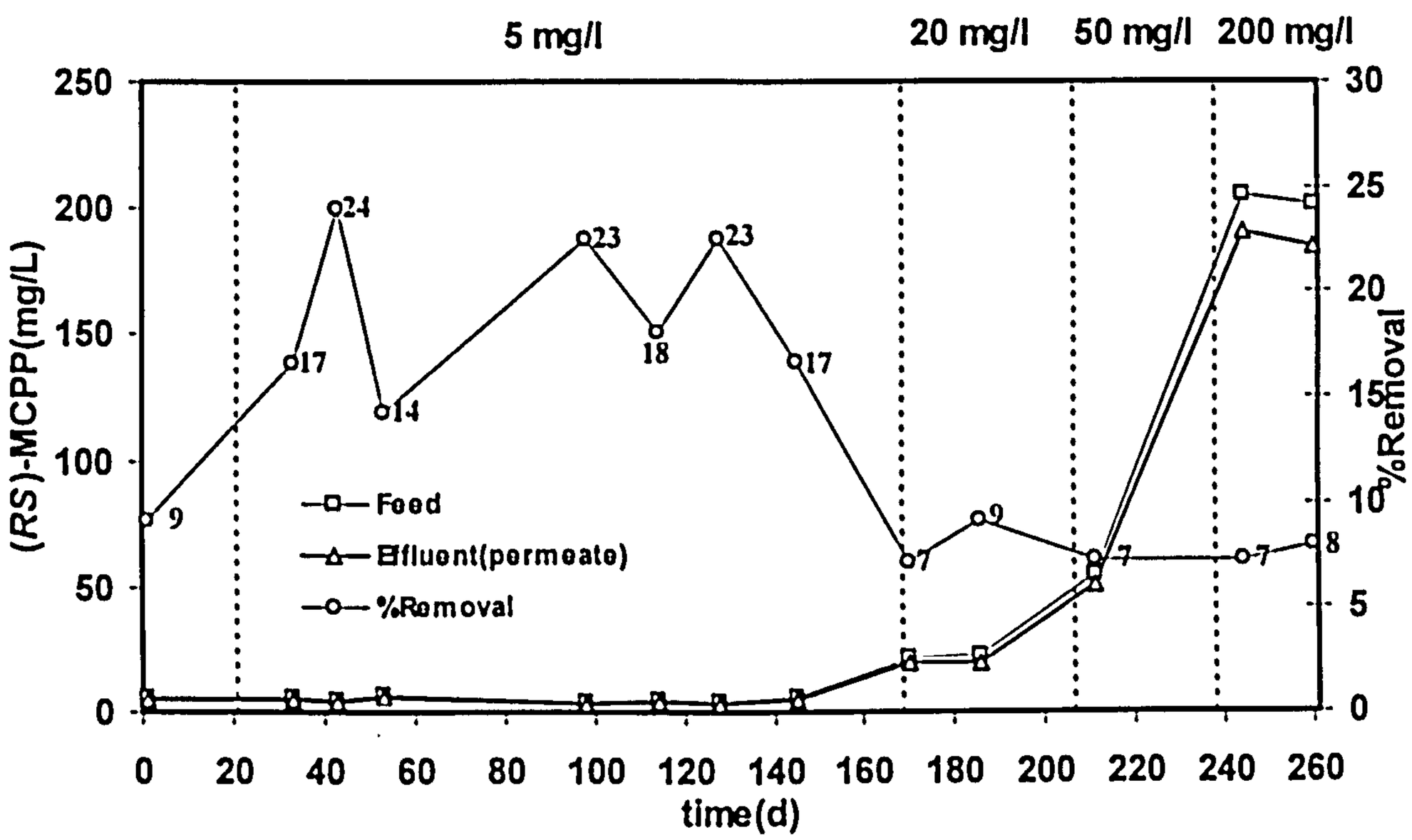


Figure 5.7: (*RS*)-MCPP degradation in the AMBr during Phase I under different (*RS*)-MCPP feed concentrations.

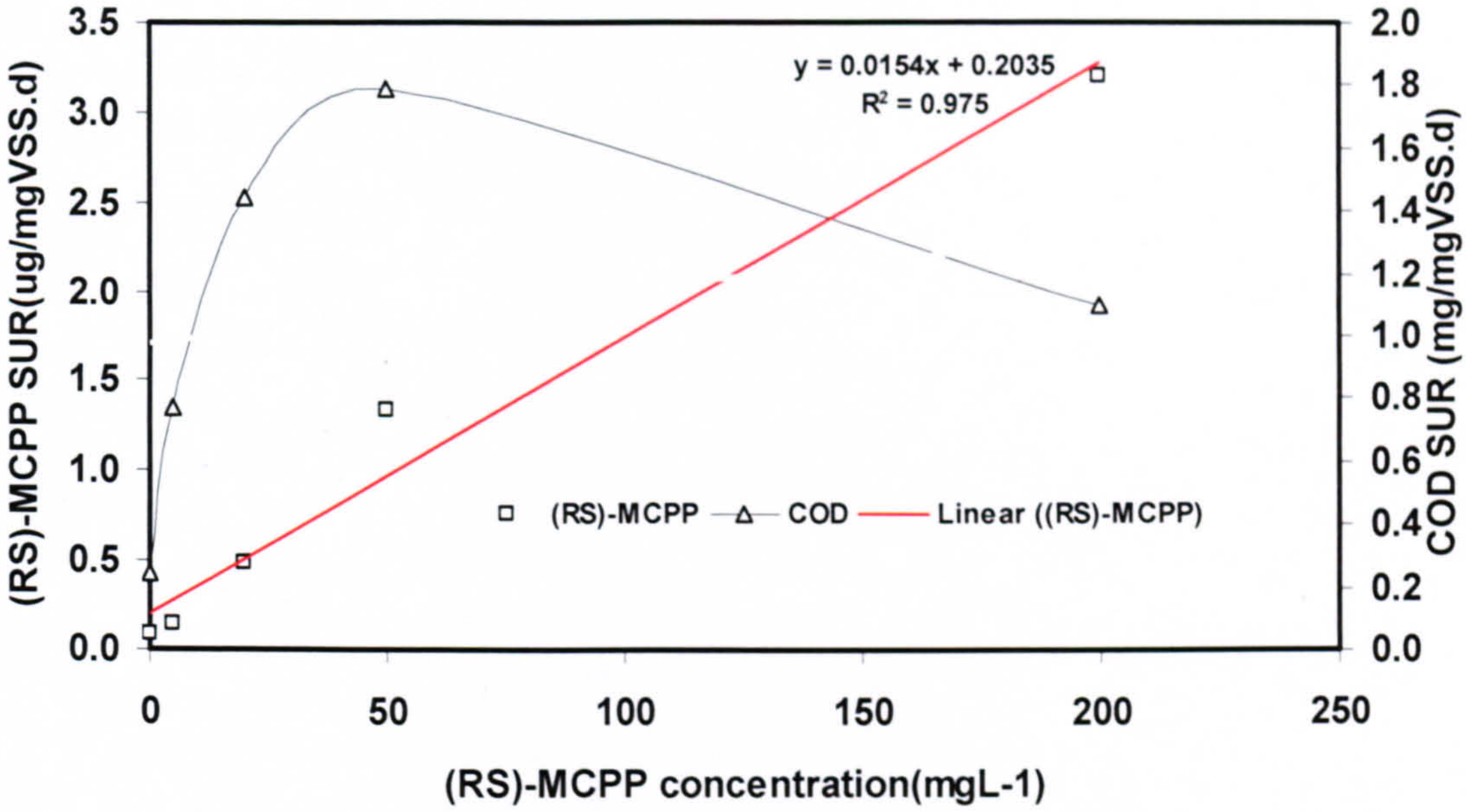


Figure 5.8: Specific COD and (*RS*)-MCPP utilisation rates in the AMBr under different under different (*RS*)-MCPP feed concentrations.

5.3.7 MLVSS & MLSS

There was no biomass washout detected throughout Phase I which is one of the advantages of using an AMBr compared to other reactor systems. The effectiveness of the polyethersulphone (PES) hollow fibre membrane with a nominal pore size of 0.5 μm in retaining biomass and producing a clear effluent resulted in the loss of biomass from the bioreactor which was no greater than 60 $mg.L^{-1}$ (Figure 5.9). The loss of biomass between days 112 and 168 was quite substantial due to blockage which led to the reactor being cleaned and some loss of the biomass occurring. No washout permits faster start-ups and enables greater stability towards any changes in the reactor operating system and more importantly alleviates the need for a large settling unit.

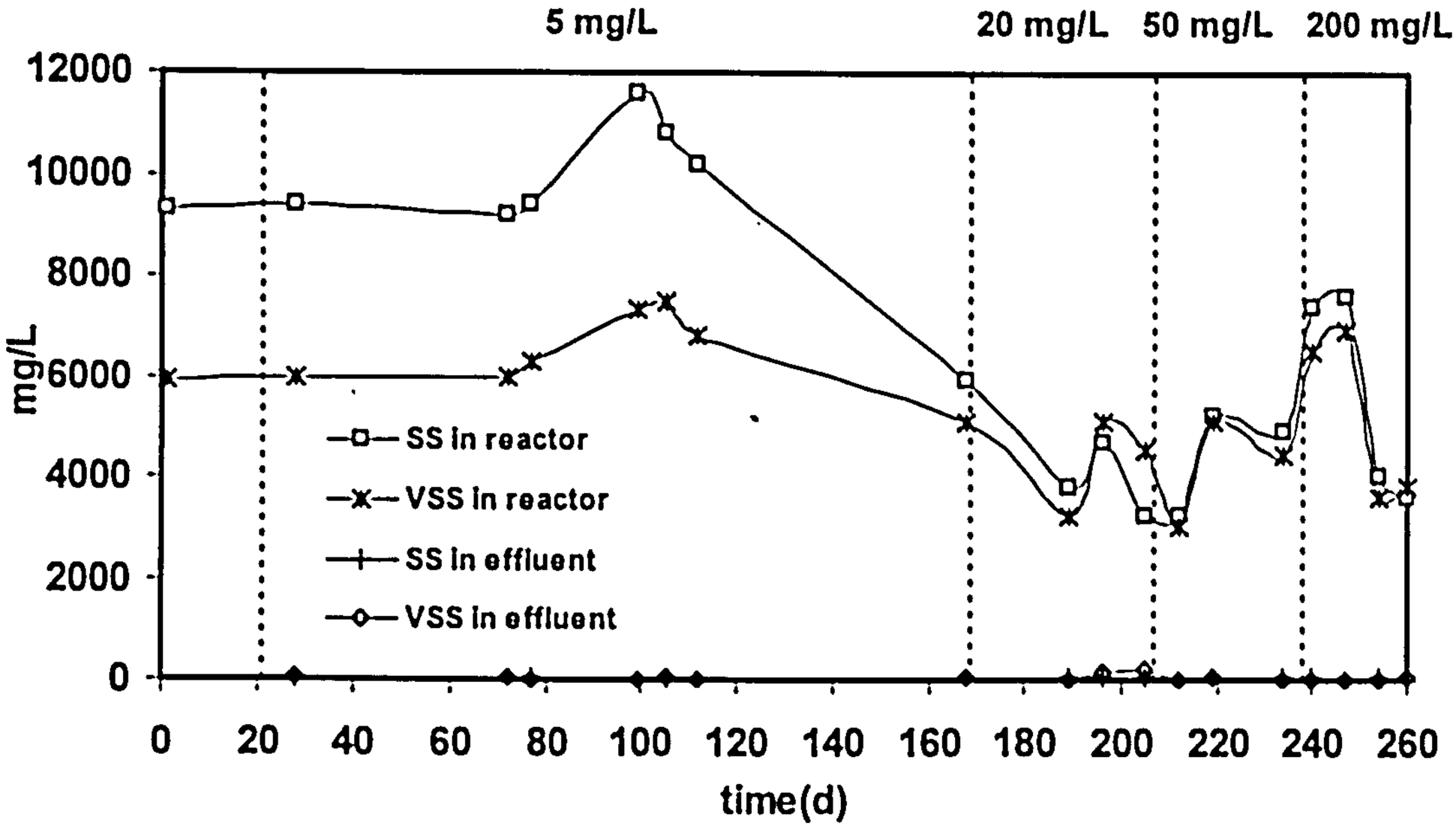


Figure 5.9: SS and VSS of mixed liquor and effluent (permeate) in the AMBr during Phase I.

5.4 CONCLUSIONS

The following conclusions can be drawn in this study:

- The methanogens were not affected by elevated (*RS*)-MCPP concentrations;
- High tolerance of potentially sensitive methanogenic populations to possible inhibitory effects of (*RS*)-MCPP was observed, with methane yields being near to the theoretical values;
- The increases in the (*RS*)-MCPP specific utilization rate that were observed over time were probably due to gradual acclimatization of bacterial populations resulting from the selection and/or evolution of strains capable of metabolising (*RS*)-MCPP;
- The long acclimatization time required to effect a substantial increase in (*RS*)-MCPP utilisation rate either suggests that the development and

selection of new or improved strains of degradative microorganisms was an inherently slow process, or that these processes were possibly assisted by the higher (*RS*)-MCPP concentrations used towards the end of the experiment causing greater diversification leading to the selection of strains able to utilise (*RS*)-MCPP more effectively.

CHAPTER SIX

EFFECT OF (*RS*)-MCPP DEGRADATION UNDER ANOXIC CONDITION (NITRATE REDUCING BACTERIA)

6.1 INTRODUCTION

Studies on (*RS*)-MCPP degradation using nitrate as a specific terminal electron acceptor using AMBr under anaerobic conditions has not been reported in the literature. However, studies using laboratory microcosms have shown that (*RS*)-MCPP is able to degrade under aerobic conditions (Rugge et al, 2002) but only limited studies have demonstrated degradation under nitrate reducing conditions (Harrison et al, 2003; Larsen & Aamand, 2001). Under aerobic conditions, redox potential is high; up to 800 mV (Doong et al., 1996) and O_2 will be used as an electron acceptor.

Nitrate reduction under anaerobic conditions utilises nitrate instead of oxygen as a terminal electron acceptor. This reaction has higher redox potential (up to 432 mV) (Klüber & Conrad, 1998) compared to other reduced conditions such as methanogenesis (< - 200 mV), hence the presence of nitrate might promote better degradation of (*RS*)-MCPP by denitrifiers. There are three types of microbial nitrate reduction (Tiedje, 1988): denitrification (1), nitrate assimilation (2) and dissimilatory nitrate reduction to ammonium or DNRA (3).

$$5(\text{organic-C}) + 2H_2O + 4NO_3^- \longrightarrow 2N_2 + 4OH^- + 5CO_2 \quad (1\ \&\ 2)$$

$$NO_3^- + 2H^+ + 4H_2 \longrightarrow NH_4^+ + 3H_2O \quad (3)$$

In this study, the objectives were to investigate (*RS*)-MCPP degradation in an anaerobic process using nitrate as an available electron acceptor, to evaluate the effects of denitrifying conditions on (*RS*)-MCPP degradation and investigate the influence of the COD/N-NO_3^- ratio on methanogenic and denitrifying activities in a single reactor unit.

6.2 METHODS

An AMBr was used in this experimental phase (Phase II) and was operated as a continuation of the study into the effect of elevated (*RS*)-MCPP concentrations under methanogenic conditions (Chapter 5). A synthetic wastewater identical to the one described in Chapter 5 except that nitrate was added to the influent in the form of potassium nitrate, was used in order to encourage the growth of denitrifying bacteria in the system.

During Phase II, the reactor was operated at COD/N-NO_3^{-1} ratios 250, 8, 3, 1, 0.3 and 0.2; and these were achieved in three ways; (i), adding 20(±6) and 600(±148) mg.L^{-1} of potassium nitrate to the feed which gives corresponding COD/N-NO_3^{-1} ratios of 250 and 8 respectively, (ii), reducing the COD of beer waste concentration from 5000(±370) mgCOD.L^{-1} to 3500(±395) mgCOD.L^{-1} in tandem with increased potassium nitrate concentration to 1100(±160) mg.L^{-1} (COD/N-NO_3^{-1} ratio 3), and iii) the final ratio was achieved by decreasing COD of the beer waste from 3500(±395) mgCODL^{-1} to 1500(±230), 300(±15) and 200(±70) mgCOD.L^{-1} at a potassium nitrate concentration of 1100(±160) mg.L^{-1} which gives corresponding COD/N-NO_3^{-1} ratios of 1, 0.3 and 0.2 respectively.

As a result of this loading regime, the reactor was operated with step decreases in organic loading rate (OLR) from 1.51(±0.1) $kgCOD.m^{-3}.d^{-1}$ to 0.07(±0.2) $kgCOD.m^{-3}.d^{-1}$ and was maintained at a constant hydraulic retention time (HRT) of 3.3 d (Table 6.1). Throughout the study, pH, COD VFA, biogas production, (*RS*)-MCPP degradation, MLVSS and MLSS, and nitrate reduction were measured according to standard methods and full details of sampling and analytical procedures are mentioned in section 4.6.

Table 6.1: Summary of reactor operational of AMBr system in Phase II.

OLR[a]	COD/ NO_3^--N	Day	HRT (d)	(*RS*)-MCPP[b]	Influent COD[c] (mgL^{-1})	Nitrate (mgL^{-1})
1.51	250	263-294	3.3	200	5000	20
1.51	8	295-312	3.3	200	5000	600
1.05	3	313-333	3.3	200	3500	1100
0.45	1	334-359	3.3	200	1500	1100
0.09	0.3	360-394	3.3	200	300*	1100
0.07	0.2	395-420	3.3	100	200	1100

[a]= average value ($kg.COD.m^{-3}.d^{-1}$), [b]= mgL^{-1}, [c]= average COD
*COD provided entirely by the 200 $mg.L^{-1}$ (*RS*)-MCPP (no other carbon added)

6.3 RESULTS & DISCUSSION

6.3.1 pH

The pH of the reactor and effluent followed a similar pattern and started to increase gradually from pH 6.8 to 7.2 soon after 20(±6) $mg.L^{-1}$ of nitrate (COD/N-NO_3^{-1} ratio of 250) was added (Figure 6.1). This was due to elevated hydroxyl ion (OH^{-1}) production during denitrification (Equation 1 & 2). During denitrification, pH levels increased (Figure 6.1) according to stoichiometry by an average of 0.25 units (Barber & Stuckey, 2000). According to Glass & Silverstein (1997), an increase in pH is a clear indicator that the denitrification

reaction is progressing. A similar finding was obtained by Akunna et al., (1994) at the end of their experiment when all culture media which received nitrate had higher pH values than that of the blank culture medium.

Moreover, on day 295 when the COD/N-NO_3^{-1} ratio was decreased to 8, pH levels dropped transiently before fluctuating at pH 7.0(±0.03) but then increased rapidly to pH 7.3 after 13 days at the ratio of 8. However, when the COD/N-NO_3^{-1} ratio was decreased to 3, the pH levels started to fluctuate again before risings tightly to pH 7.3(±0.07). Decreasing the ratio further to 1 brought a rapid increase in pH levels from pH 7.6 to pH 8.3 due to the accumulation of more alkalinity (OH^{-1}) in the system. The pH levels then remained fairly stable (pH 8.3(±0.11)) at COD/N-NO_3^{-1} ratios of 0.3 and 0.2 despite the fact that during the later stage of this experiment only (*RS*)-MCPP was present in the influent as carbon a source (day 360 to 395).

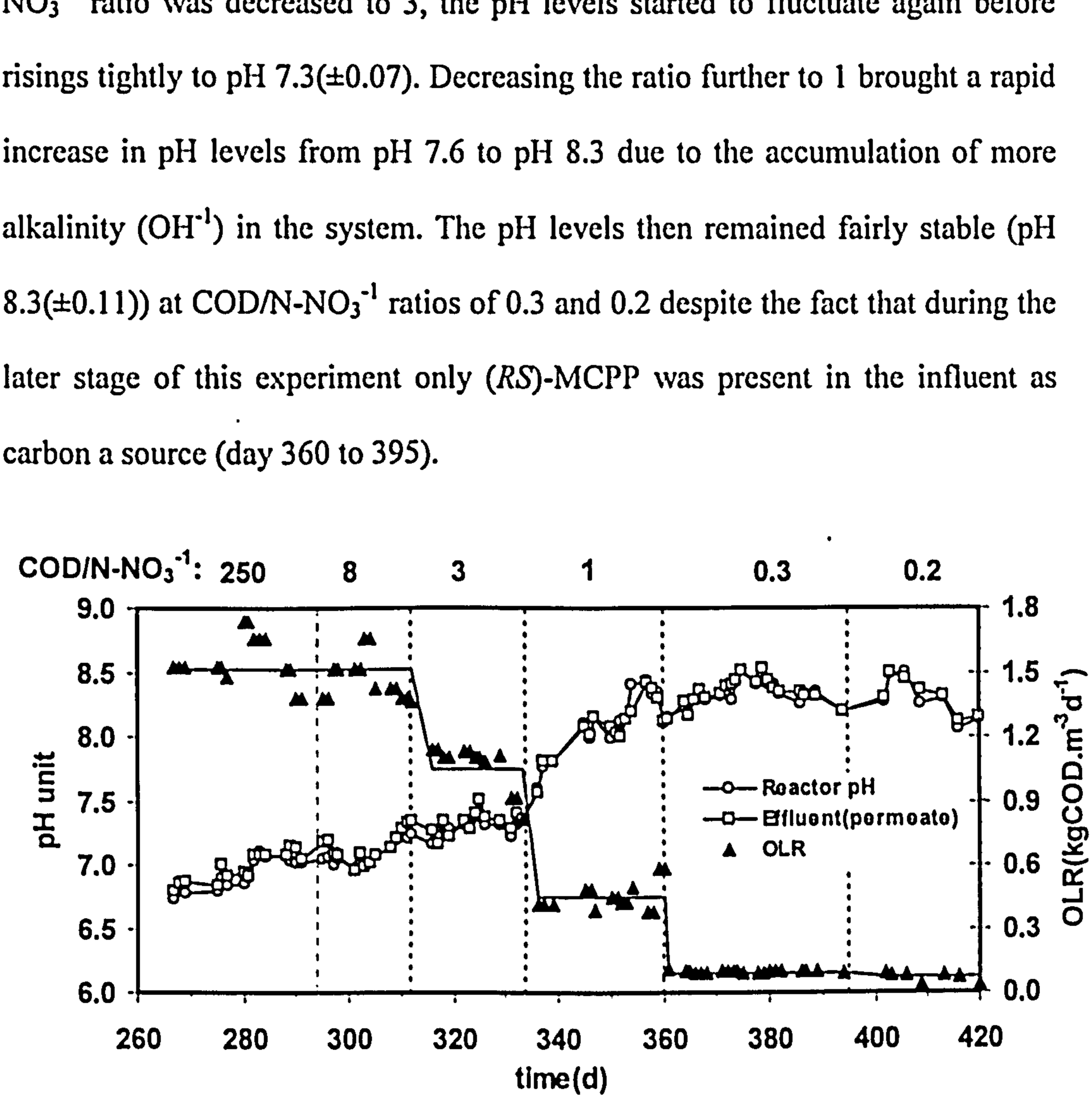


Figure 6.1: pH of the AMBr under different COD/N-NO_3^{-1} feed ratio.

6.3.2 COD Removal

The soluble COD removal efficiency at COD/N-NO_3^{-1} ratios of 250 and 8 was relatively constant and had an average removal over both ratios of 93(±0.6)% (Figure 6.2). However, the soluble COD removal efficiency showed a slight reduction from this value to an average removal of 90(±1.25)% at a ratio of 3. Decreasing the COD/N-NO_3^{-1} ratios further to 1 by reducing OLR from 1.05(±0.1) to 0.45(±0.07) kgCOD.m^{-3}.d^{-1} reduced the soluble COD removal efficiency further to 83(±3)%.

Unlike the pattern of methane production (Figure 6.5) which gradually declined over time starting from a COD/N-NO_3^{-1} ratio of 8, the soluble COD removal efficiency was eventually maintained at a value above 80% (up to COD/N-NO_3^{-1} ratio of 3) for 92 days. This indicates that the reduced fraction of COD eliminated via methanogenic pathways in Phase II was compensated for by a higher degree of COD removal from the denitrification process, thereby maintaining a similar overall COD removal efficiencies and thus is in agreement with the statement that methanogenic activities begin only after denitrification is completed (Akunna et al., 1992; Moquera-Corral et al., 2001; Sponza & Atalay, 2004), and that denitrification is preferred to methanogenesis, as long as nitrates or nitrites are present (Chen et al., 1993; Bollag & Czlonkowski, 1973; Balderston & Payne, 1976; Hanaki & Polprasert, 1989).

At an OLR 0.09(±0.004) kgCOD.m^{-3}.d^{-1} (COD/N-NO_3^{-1} ratio of 0.3), the soluble COD removal efficiency declined rapidly to 27% within 21 days (day 382)

before further dropping to 6% removal after a further 12 days (day 394). This poor COD removal efficiency may have been due to the OLR being reduced rapidly by fivefold from 0.45(±0.07) to 0.09(±0.004) kg.COD.m^{-3}.d^{-1} which caused insufficient substrate to be available for the microbial population in the system, leading to system failure. In addition, only complex carbon sources provided by (*RS*)-MCPP (100% carbon source in feed) were available for bacteria as an organic substrate compound or electron donor and this may have caused a high level of cell mortality, a theory supported by the reduction in biomass from 2800 to 1100 mg.L^{-1} at this time (Figure 6.10). However, the system showed a quick recovery when beer waste was introduced back into the influent (to maintain the OLR of the influent after (*RS*)-MCPP had been reduced from 200(±3) mg.L^{-1} to 100 mg.L^{-1}). Consequently, the soluble COD removal efficiency increased rapidly to 66% by day 402 and further improved to 93% by day 413 before gradually declining to 90% on day 420.

In comparisons between the use of nitrate and CO_2 as electron acceptors, nitrate was found to result in higher bacterial yields from carbohydrates (0.534 gVSS.g^{-1} compared to 0.208 gVSS.g^{-1} for nitrate and CO_2 as electron acceptors, respectively), and a slower generation time required for a doubling in bacterial population (16 times faster with nitrate as electron acceptor compared to CO_2, Barber &Stuckey, 2000). Hence, increases in reactor COD (between COD/N-NO_3^{-1} ratios of 8 and 3 - Figure 6.3) might be caused by new biomass being rapidly produced and higher utilisation associated products (UAP) being formed.

However, at a COD/N-NO_3^{-1} ratio of 1, the reactor COD started to decline coinciding with a drop in nitrate removal efficiency (Figure 6.11) suggesting that the denitrification activity dropped and affected the generation of the bacterial populations when nitrate was the predominant electron acceptor.

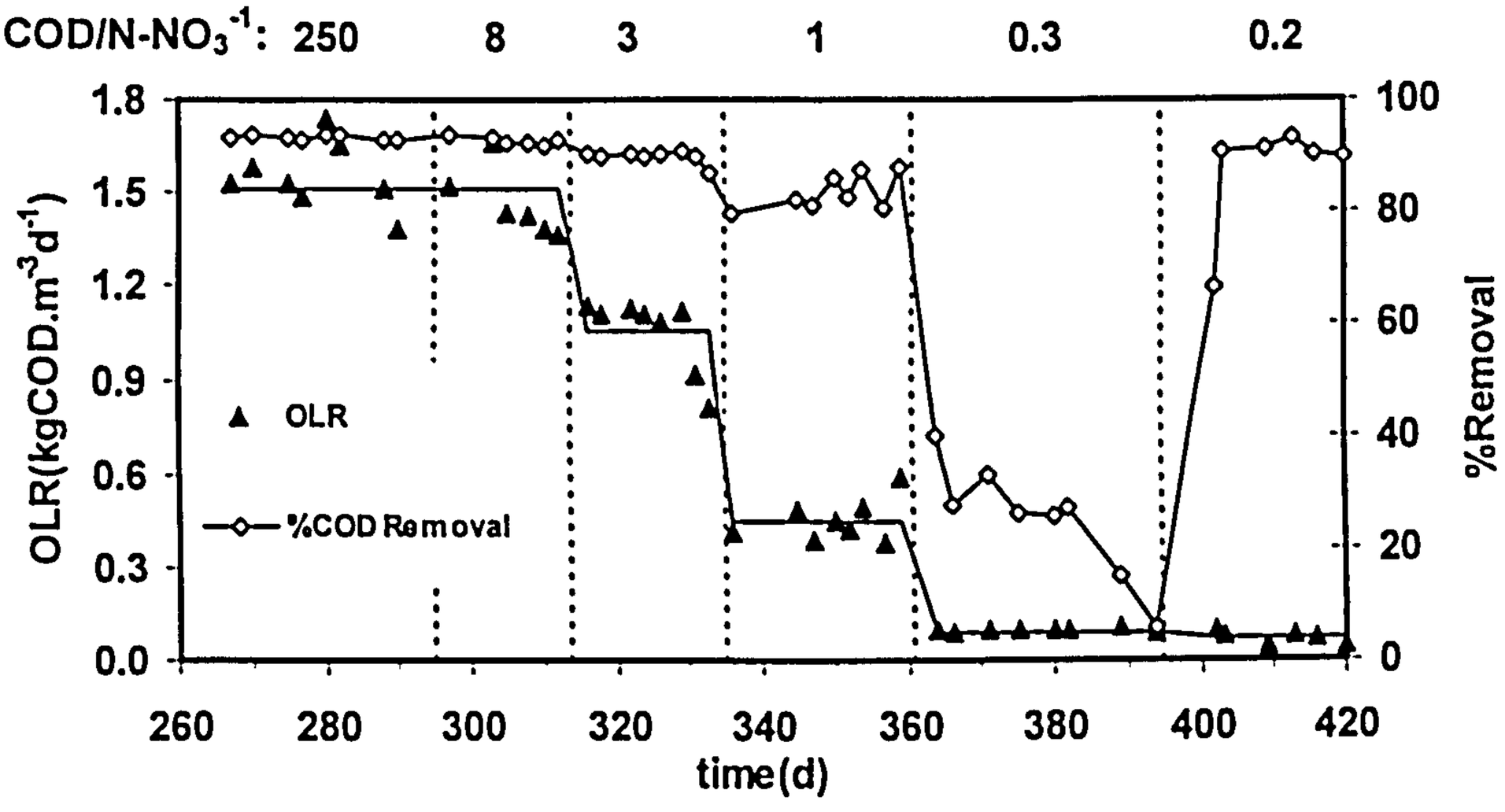


Figure 6.2: COD removal efficiency of the AMBr under different COD/N-NO_3^{-1} feed ratios.

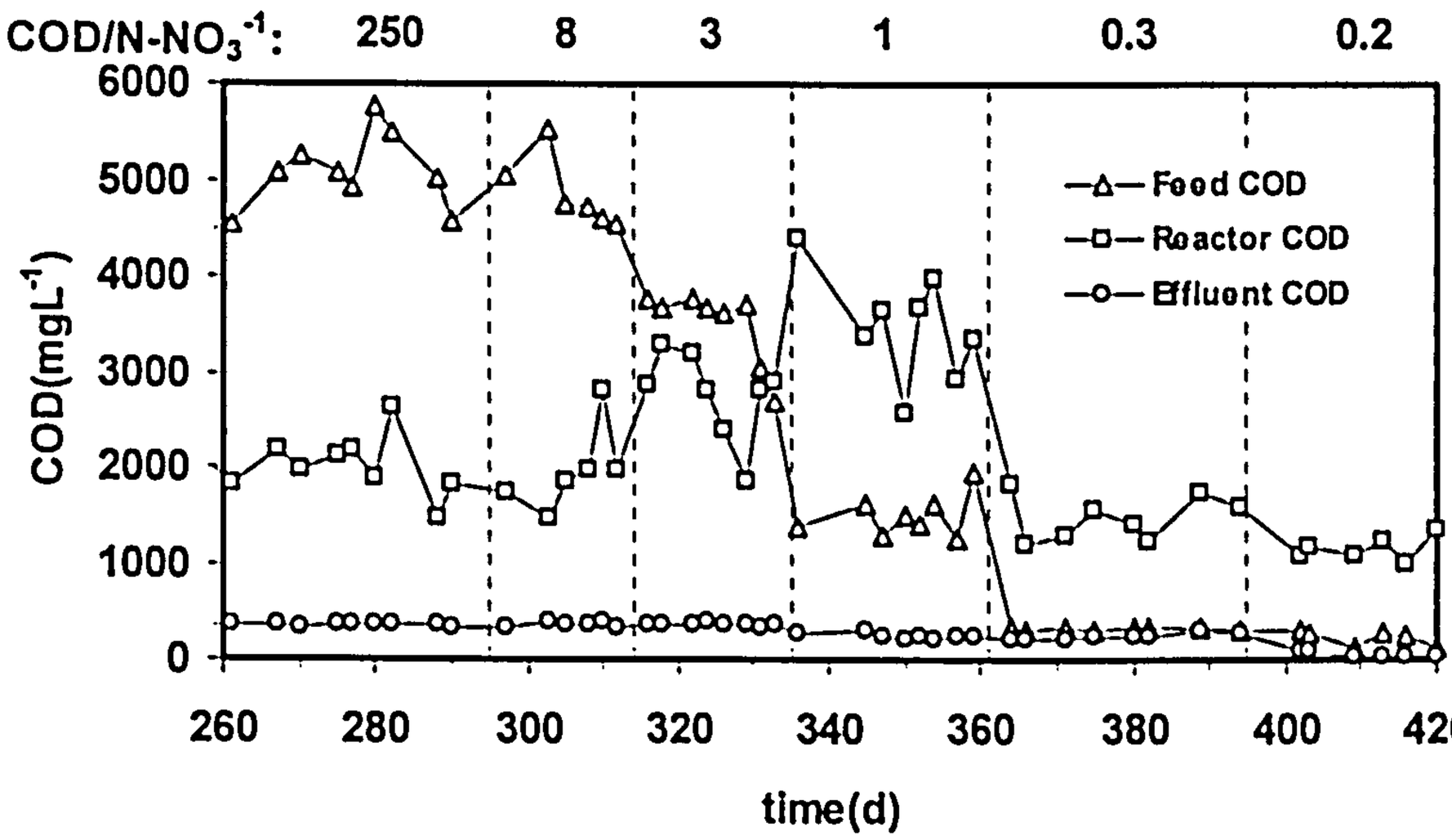


Figure 6.3: COD of the AMBr under different COD/N-NO_3^{-1} feed ratios.

6.3.3 VFA

Denitrifiers have high affinity for acetate as an electron donor (Akunna et al, 1993) and improved acetate utilisation during denitrification is likely to be caused by acetotrophic denitrifiers (Barber & Stuckey, 2000) which further explain the low methane production by methanogenesis (observed at days 337 – 416 in Figure 6.5). However, 16 days after the OLR was decreased from 1.51 $kgCOD.m^{-3}.d^{-1}$ to 1.05 $kgCOD.m^{-3}.d^{-1}$, (at a COD/N-NO_3^- ratio of 3) an increase in acetate concentrations (from 7.12 $mg.L^{-1}$ to 47.74 $mg.L^{-1}$) was observed in the system.

It was anticipated that propionate degradation would be influenced by hydrogen levels in the system (Barber & Stuckey, 2000). Even though hydrogen concentration was not measured in the current study, high hydrogen demand of dissimilatory nitrate reduction to ammonium (Equation 3), suggests reduced

hydrogen levels would be present in the reactor under nitrate reducing conditions. This being the case, improved environmental conditions would have prevailed for efficient propionate degradation since it is critically influenced by hydrogen levels (Barber & Stuckey, 2000), and was further demonstrated in Figure 6.4 when low propionate concentrations were detected (below detection levels) during this phase.

The observation of low butyrate concentrations (below 13 $mg.L^{-1}$) coincided with previous work (Barber & Stuckey, 2000) and was in agreement with McCarty and Mosey's (1991) hypothesis that butyrate is produced under stress to counteract the excessive decreases in pH; this was not observed in the current study (Figure 6.1) presumably because denitrification did not increase the stress on the microbial communities in the AMBr.

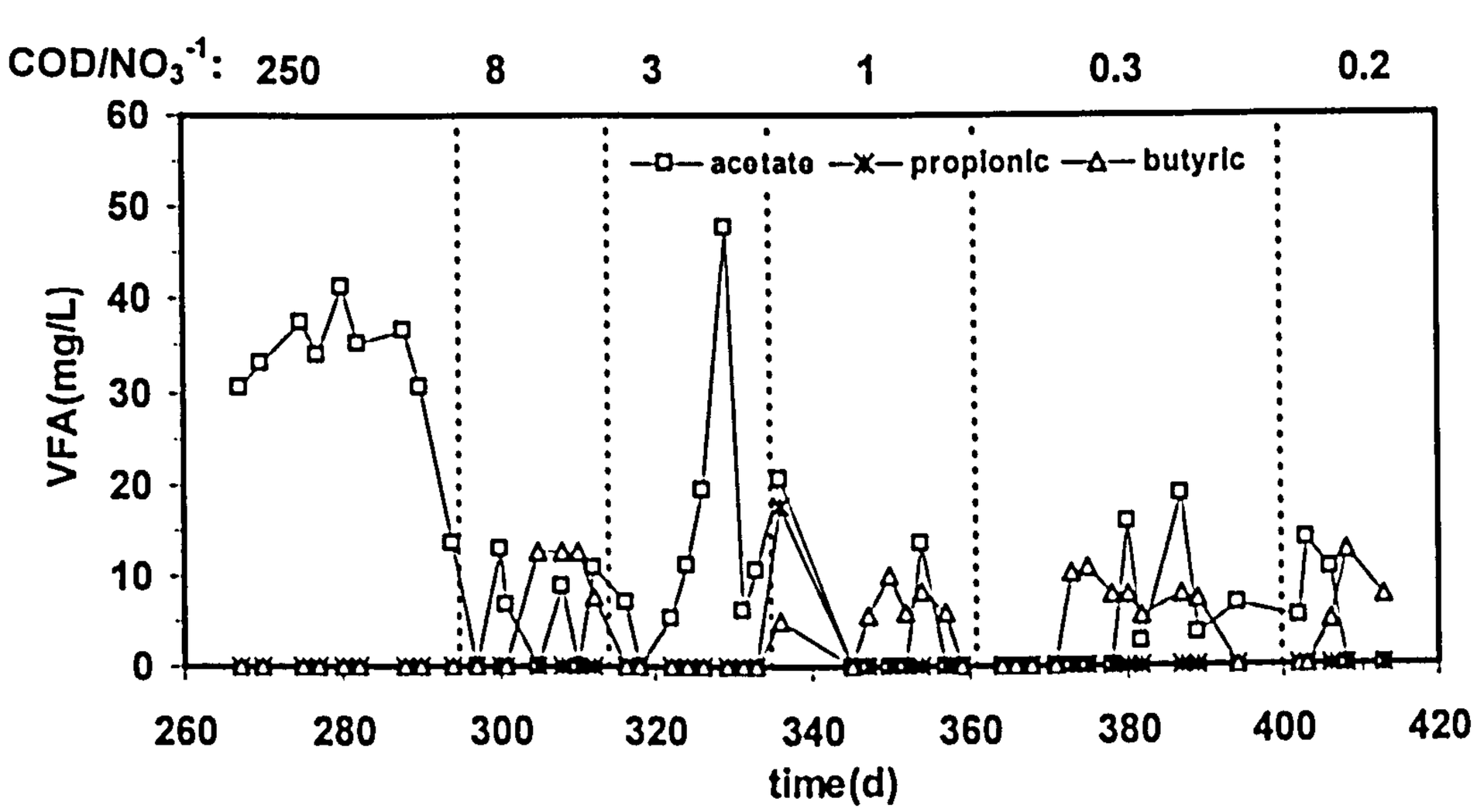


Figure 6.4: VFA profile of the AMBr under different COD/N-NO_3^{-1} feed ratios.

6.3.4 Biogas

Methane production was stable at a COD/N-NO_3^{-1} ratio of 250, maintaining an average of 13.4 $L.d^{-1}$, but started to decline gradually at a COD/N-NO_3^{-1} ratio of 8 on day 301 (6 days after the COD/N-NO_3^{-1} ratio of 8 was introduced) from 12.65 $L.d^{-1}$ to below detection level after 100 days at a ratio of 0.2 (Figure 6.5). Since the feed COD was not the same, this phenomenon was expected because of the reductions in OLR over time and concomitant increases in N-NO_3^{-1}, making relatively lower amounts of COD available for methane production since methanogenesis could only commence after the total reduction of nitrates by denitrifiers. Thus the reduction in methanogenic activity due to the presence of nitrate was caused by higher affinity of denitrifier towards organic carbon compared to methanogens since the current AMBr system used in the study is a continuous system.

According to Barber & Stuckey (2000), Equation 3 is as equally favourable to mixed bacterial consortia as nitrate reduction to nitrogen (Equations 1 & 2). As a result of dissimilatory nitrate reduction to ammonium (DNRA-Equation 3), ammonium ions are produced. Although this will enhance the availability of reduced nitrogen as a nutrient for methanogens, high concentrations can lead to operational failure and free ammonia levels should be kept below 80 $mg.L^{-1}$ (Anderson et al., 1982). Thus, accumulation of ammonium ions through DNRA might be another explanation for the methanogenesis inhibition observed at low COD/N-NO_3^{-1} ratio (COD/N-NO_3^{-1} ratio of 1, 0.3 and 0.2).

Another possible inhibitory effect of methane production by the presence of nitrate was suggested due to the toxic effect of enzyme inhibition and/or changes in the redox potential (Chen & Lin, 1992). According to Klüber & Conrad (1998), micromolar concentrations of nitrite were sufficient to completely inhibit methanogenesis and even though nitrite was not detected in the system until day 382 at concentration 1 $mg.L^{-1}$ it soon increased rapidly to 40 $mg.L^{-1}$ after 12 days (Figure 6.12). However, Clarens et al., (1998) proposed that the growth of denitrifying organisms (carbon competition) was responsible for the methane production failure rather than chemical inhibition by nitrite itself.

In contrast, the methane gas percentage was stable at 80(±2) % for the reactor over COD/N-NO_3^{-1} ratios of 250, 8 and 3 (Figure 6.6). The methane gas percentage observed in this study is generally higher than other reported values. For example, in their study of the laboratory-scale completely-stirred anaerobic digesters fed with synthetic wastewaters, Akunna et al., (1992) reported only 55% at a COD/N-NO_3^{-1} ratio of 53 and further concluded that varying COD/N-NOx^{-1} ratio brought about to three zones: methanisation only (COD/N-NOx^{-1} ratio > 53), methanisation and denitrification (8.86 ≤ COD/N-NOx^{-1} ratio ≤ 53) and denitrification only (COD/N-NOx^{-1} ratio < 8.86). However, different sources of wastewater and operating conditions dictate the methane content in the biogas (refer to Section 5.3.5). Akunna et al (1992) used synthetic wastewater which contained mainly glucose, but the synthetic wastewater used in this current study was brewery wastewater which is likely to have contained comparatively higher concentrations of amino acids and this could have produced a higher methane composition (Speece & McCarty, 1964).

The methane percentages only started to decline rapidly at a COD/N-NO_3^{-1} ratio of 1 and below; from 77% at day 333 to 22% at day 352 before increasing again to 42% at day 361. A similar response was observed for methane yield (Figure 6.7) when a severe decline was seen only at COD/N-NO_3^{-1} ratios of 1 and below due to all possible factors mentioned earlier.

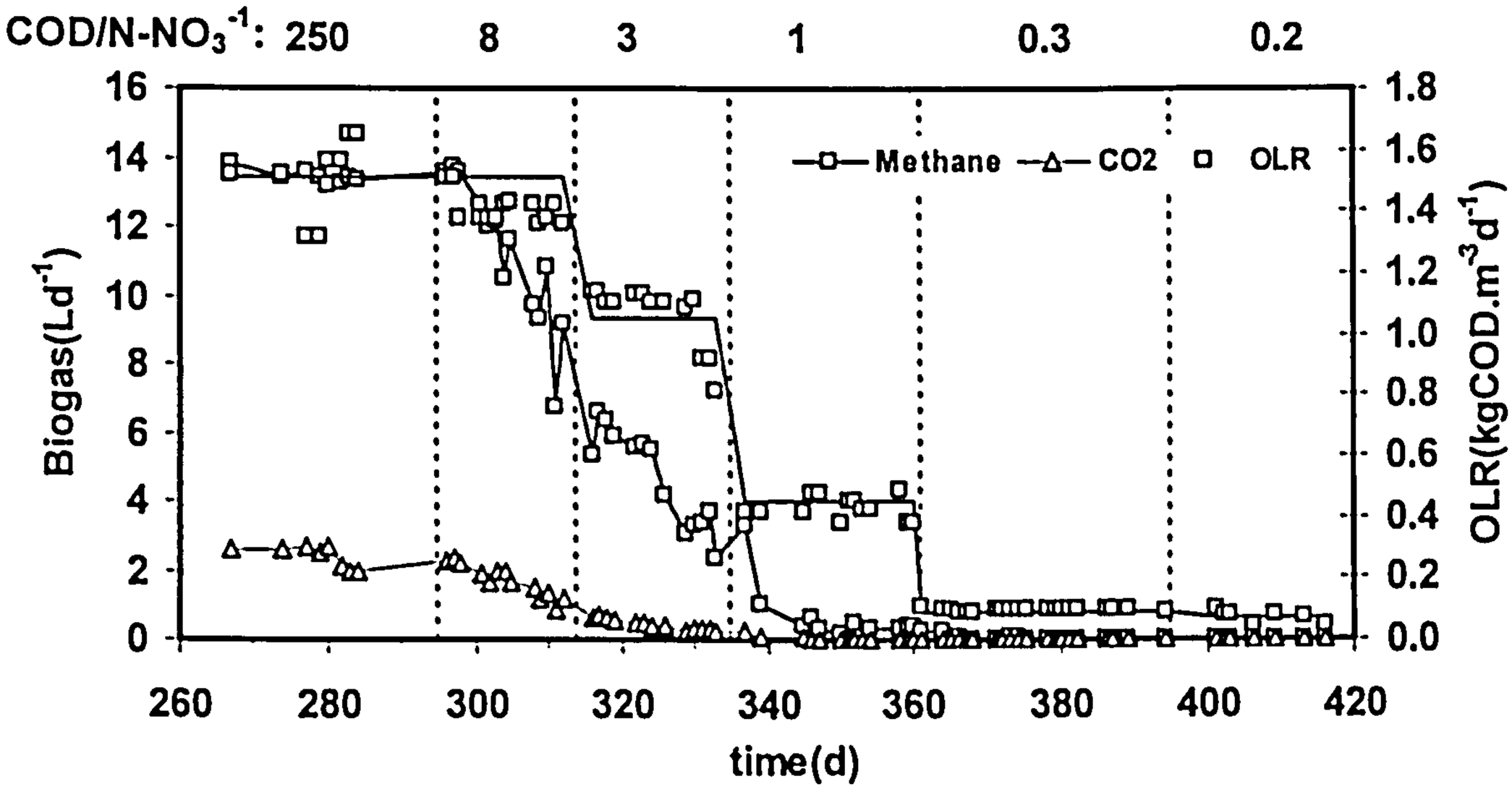


Figure 6.5: Biogas production of the AMBr under different COD/N-NO_3^{-1} feed ratios.

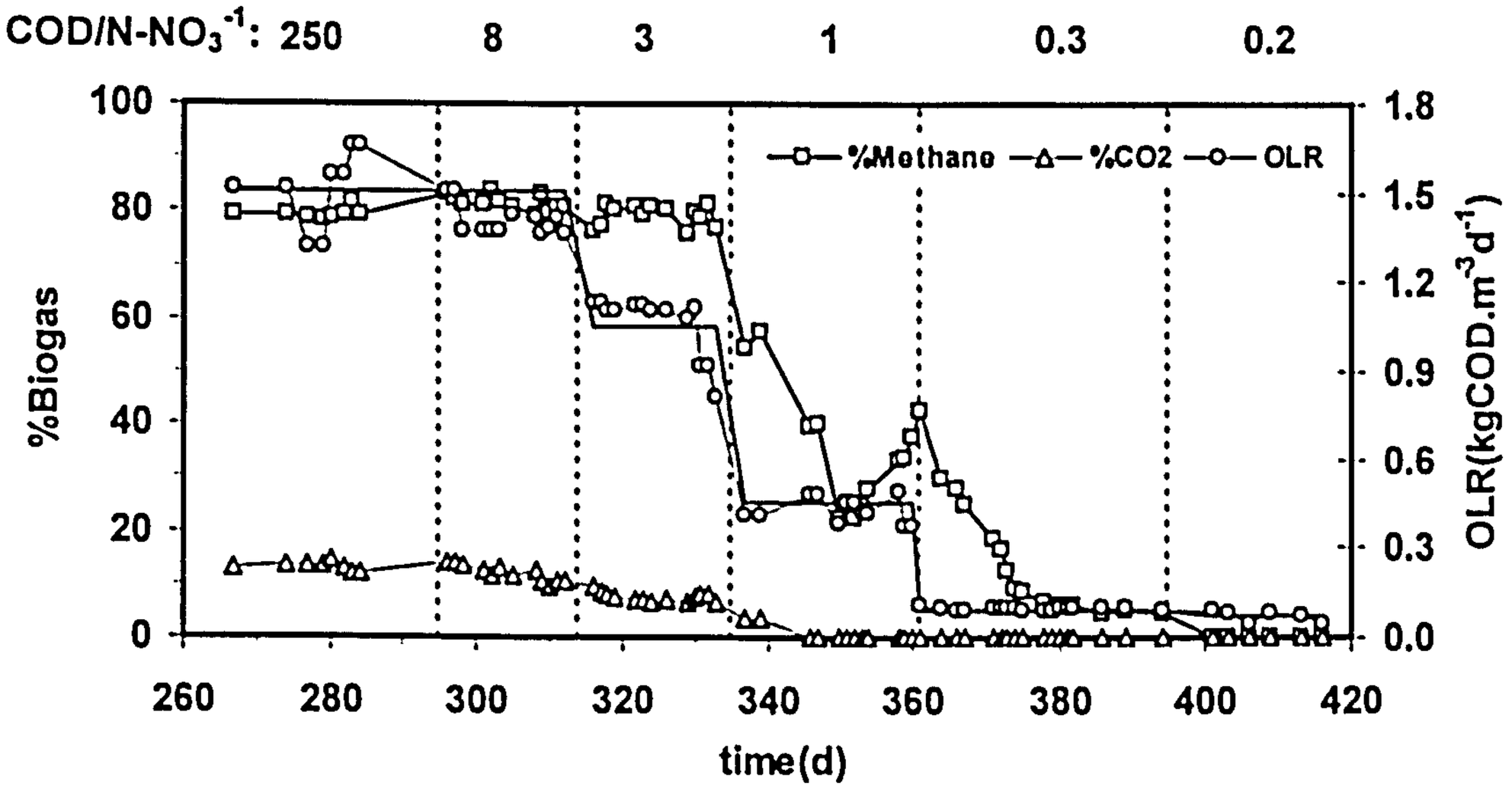


Figure 6.6: Biogas percentage of the AMBr under different COD/N-NO_3^{-1} feed ratios.

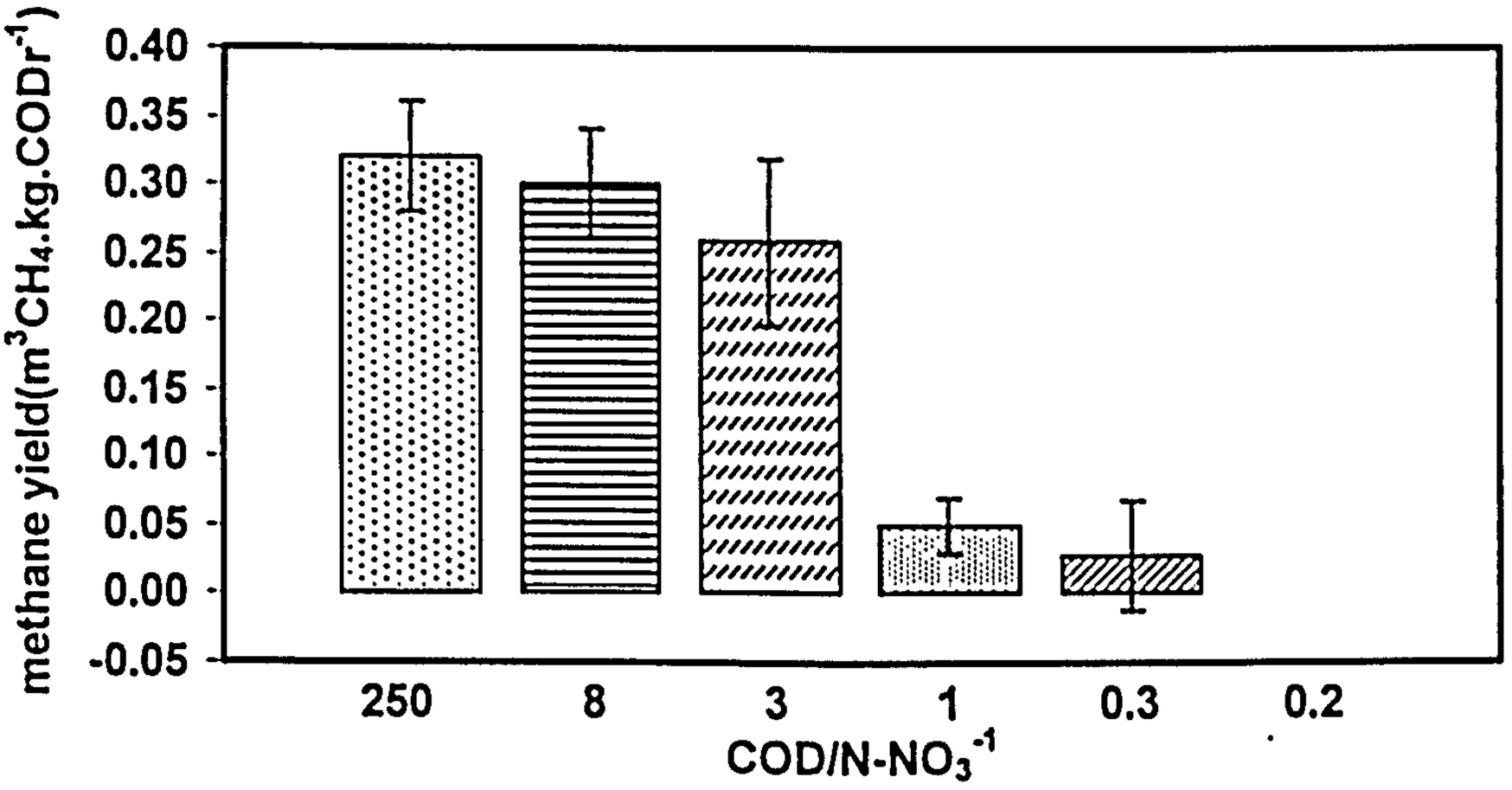


Figure 6.7: Mean methane yield of the AMBr under different COD/N-NO_3^{-1} feed ratios.

6.3.5 (*RS*)-MCPP

The (*RS*)-MCPP concentrations in the influent were maintained at 200-250 $mg.L^{-1}$ throughout Phase II, with removal efficiency increasing over time

throughout the study (increasing from 2 % to 47 % - Figure 6.8). Figure 6.9 showed that the specific utilisation rate (SUR) of (*RS*)-MCPP was inversely proportional to the COD/N-NO_3^{-1} ratio suggesting that nitrate addition had influence in (*RS*)-MCPP specific utilisation rate. In contrast, SUR of COD was directly proportional to the COD/N-NO_3^{-1} ratio, indicating that the COD SUR was dependent on OLR (increasing COD/N-NO_3^{-1} ratios caused increasing OLR).

Along with the gradual increase in (*RS*)-MCPP degradation efficiency, there was a consistent unidentified peak with increasing amplitude spotted in the HPLC chromatogram which may have been the primary metabolite in the degradation pathway of (*RS*)-MCPP. Due to time and resource limitation (no mass spectrometry detector coupled to HPLC was available), it was decided not to continue further investigation of this unidentified peak.

The presence of facultative denitrifiers (arising from the population shift mentioned earlier in Section 6.3.2) along with several factors such as long acclimatization time (157 days) and selective pressure, resulted in better (*RS*)-MCPP removal efficiency in this phase. Decreases in OLR caused less simple carbon sources (from the beer waste) available, hence provides selective pressure on microbial to utilise (*RS*)-MCPP The denitrification activity rapidly dropped from 77 % to 0 % (at COD/N-NO_3^{-1} ratio of 0.3, Figure 6.11) due to insufficient levels of easily metabolised substrate (from brewery wastewater), however, during this period the (*RS*)-MCPP utilisation rate actually increased from 28 to 71 $\mu g.mgVSS^{-1}.d^{-1}$.

Another possible explanation of the overall improvement in the (*RS*)-MCPP SUR and removal efficiency that coincided with higher denitrification activity could simply be the effect of increases in the redox potential from the presence of nitrate ions in the AMBr system. Even though redox potential was not measured in the AMBr when it was supplemented with nitrate, levels of nitrate were detected in the current study during period of addition; the literature identifies that redox potentials of N-compounds are all much higher than that of CO_2/CH_4 at +432 to -50 mV (Klüber & Conrad, 1998; Gerarrdi, 2002). Harrison et al., (2003) found that nitrate addition stimulated anaerobic biodegradation of (*R*)-MCPP and reported that as (*R*)-MCPP biodegraded anaerobically, 4-CMP accumulated rapidly as a transient metabolite. In a study by Tett et al., (1994), *Alcaligenes denitrificans* was shown to grow on (*R*)-MCPP as the sole source of carbon and energy, and *Alcaligenes denitrificans* increased in culture after a lag phase of 5 hours into the growth cycle, but this report did not mention (*R*)-MCPP removal efficiency in detail. In the current study, (*R*)- and (*S*)- enantiomers cannot be separated using the HPLC procedure developed in the earlier work because different a HPLC procedure needs to be developed in order to separate these two (*R*)- and (*S*) stereoisomers. Due to lack of resources (special column and needing a chiral mobile-phase additive to mobile phase) and time (chiral derivation needed) (Snyder, 1997), it was decided not to further develop procedures for separating enantiomers.

It is not clear which parameters (nitrate addition coinciding with changed in redox potentials, selective pressure on bacteria and long acclimatization by denitrifiers) are the most significant in (*RS*)-MCPP degradation. Further

experimental studies must be undertaken to determine the relative roles played by redox potentials and acclimatization by the biomass under the denitrification process.

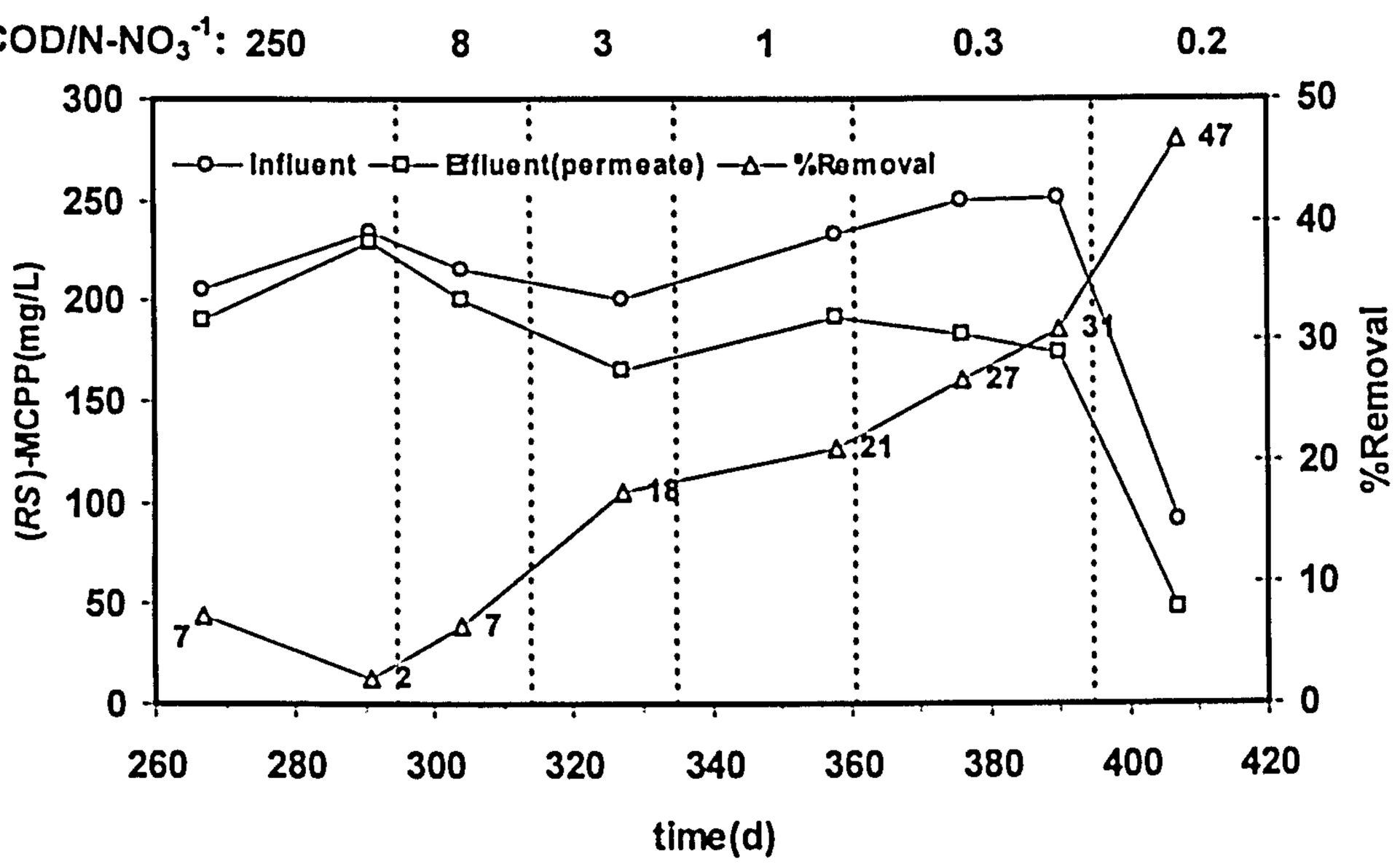


Figure 6.8: (*RS*)-MCPP degradation in the AMBr under different COD/N-NO_3^{-1} feed ratios.

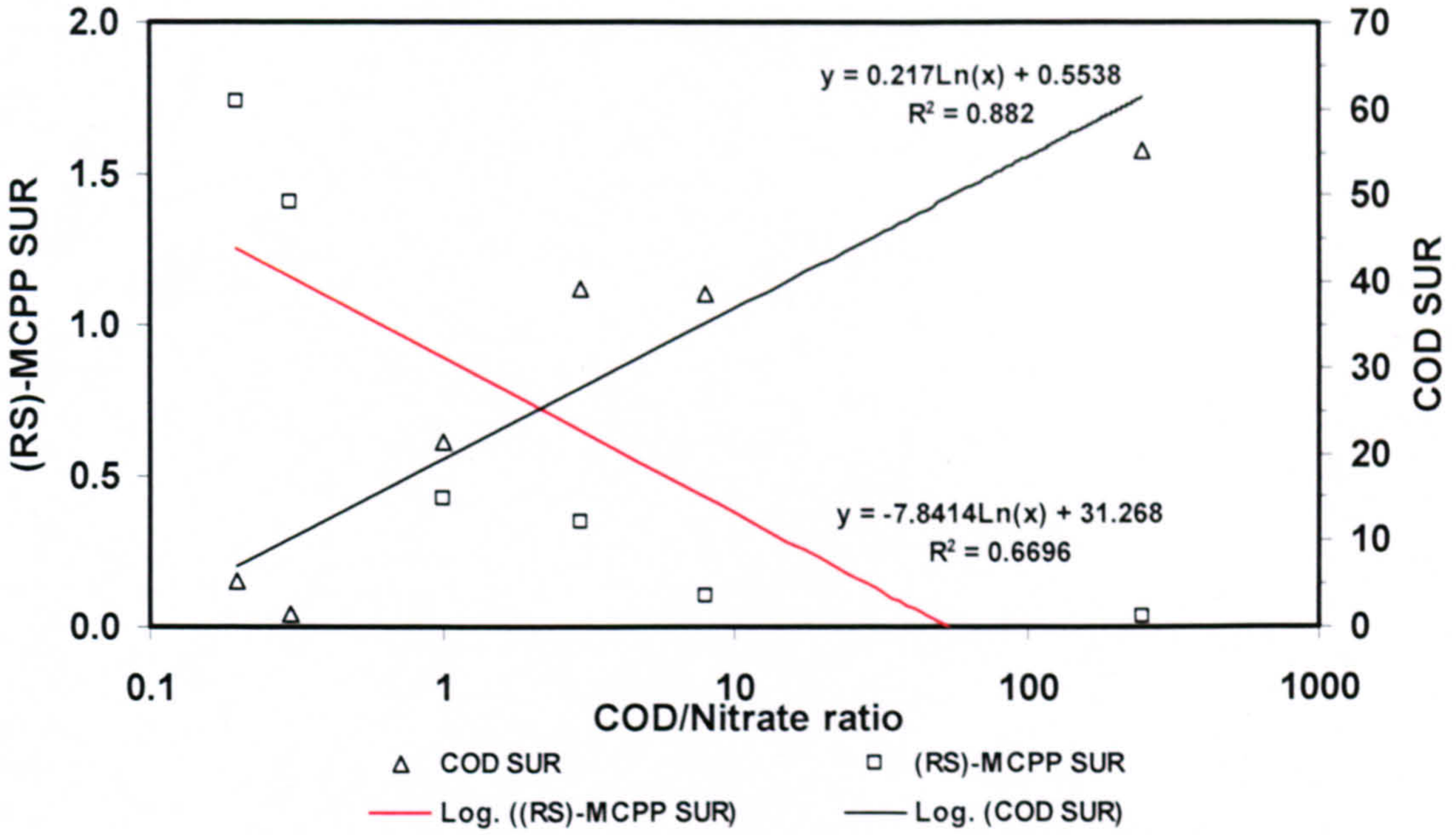


Figure 6.9: Specific COD and (*RS*)-MCPP utilisation rates in the AMBr under different COD/N-NO_3^{-1} feed ratios.

6.3.6 MLVSS & MLSS

Figure 6.10 shows that the concentrations of volatile and total suspended solids at COD/N-NO_3^{-1} ratios of 250, 8, and 3 had average values of 3789 mg.L^{-1} and 4056 mgL^{-1}, respectively. However, at a COD/N-NO_3^{-1} ratio of 1, the concentrations of VSS and TSS declined to 3000 mg.L^{-1} and 3200 mg.L^{-1}, respectively, and this was in parallel with a rapid decrease in methane composition and methane yield (Figure 6.6 & Figure 6.7) due to the OLR being reduced from 1.05 to 0.45 kgCOD.m^{-3}.d^{-1}. At this point, the methanogen population appeared to enter the death phase of the growth cycle when cells had to compete with greater numbers of denitrifiers for limited substrates.

The VSS and TSS dropped substantially at a COD/N-NO_3^{-1} ratio of 0.3, from 2800 to 1100 mg.L^{-1} due to OLR being further reduced from 0.45 to 0.09

kg.COD.m^{-3}.d^{-1} causing even greater substrate limitation for methanogens. In addition, simple organic carbon substrates were not available for cells since the feed contained (*RS*)-MCPP as the sole carbon source which was less easily assimilated than the simple COD that came from beer wastewater at high ratios. The system showed a slight recovery of sludge concentration when beer waste was returned to the influent at a ratio of 0.2 despite this condition having the lowest OLR (0.07 kgCOD.m^{-3}.d^{-1}). Very low VSS and TSS (below 50 mg.L^{-1}) were always observed in the permeate (effluent), demonstrating that the membranes were effective barriers for biomass.

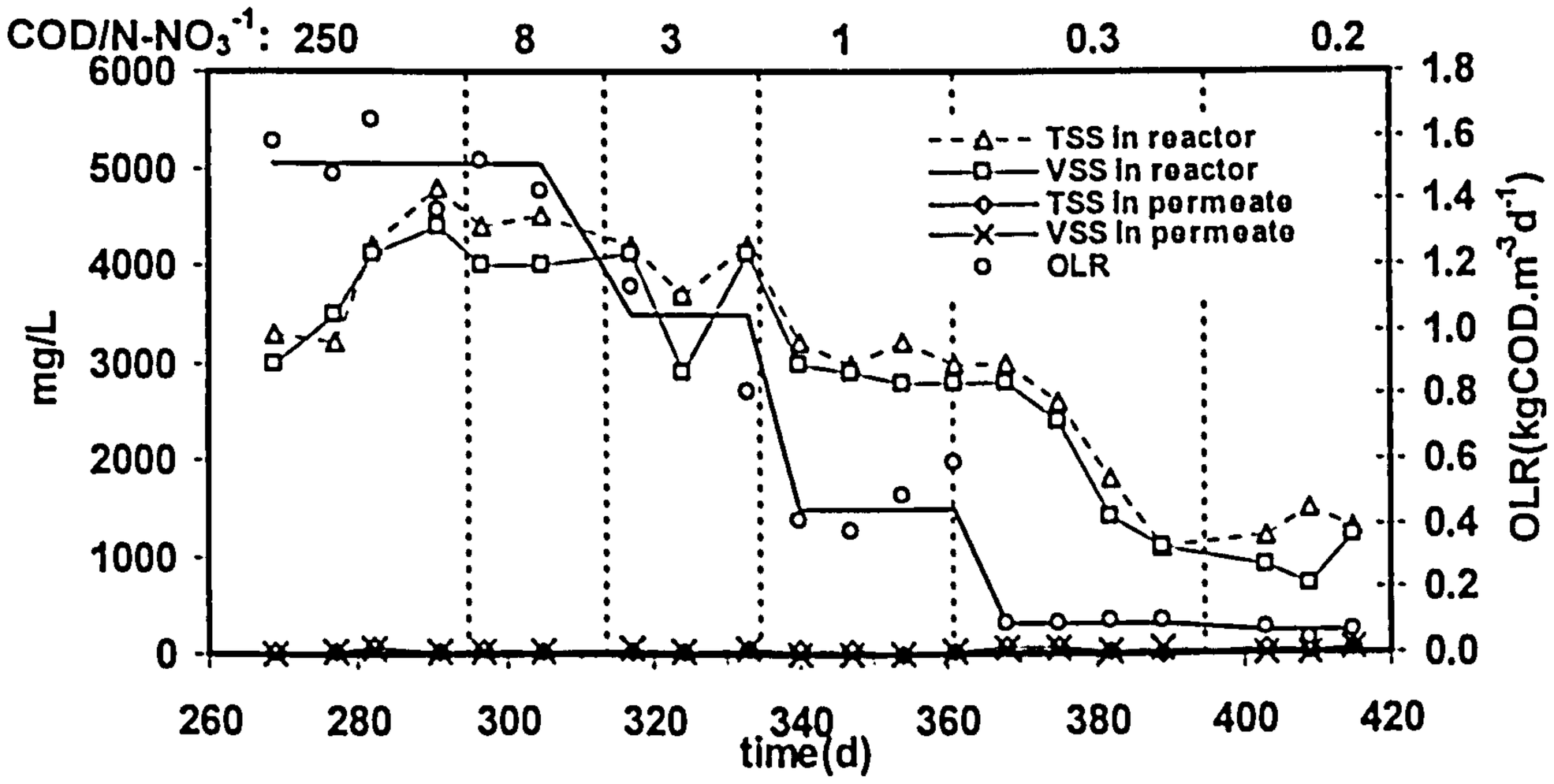


Figure 6.10: TSS and VSS of mixed liquor and effluent (permeate) in the AMBr under different COD/N-NO_3^{-1} feed ratios.

6.3.7 Nitrate reduction

Because the seed sludge had no prior exposure to nitrate (other than very low concentrations that of NH_4 that have been present in the activated sludge fed to

the sludge digester), the nitrate in the influent was initially kept at a low average concentration of 17 $mg.L^{-1}$ for the first 27 days, allowing the sludge to adapt to the presence of nitrate. However, the sludge did not take long to develop denitrification capability. When the first measurement of nitrate was carried out on day 263 (the first day after nitrate addition), the residual nitrate in the effluent was already below 0.5 mgL^{-1} (93% removal) and the removal efficiency then proceeded to increase to 99% after four weeks of nitrate dosing (Figure 6.11).

The nitrate concentration in the influent was therefore increased to 500 $mg.L^{-1}$ on day 295, and then to 1100 $mg.L^{-1}$ on day 308. Under these conditions, nearly all nitrate in the influent was denitrified (99% removal) for COD/N-NO_3^{-1} ratios of 250 down to 1; however, the removal efficiency declined sharply to only 13% at a ratio of 0.3 (Figure 6.11). At this point, poor nitrate removal was probably caused by low availability of suitable organic matter for the denitrifier due to low OLR and lack of simple carbon sources previously provided by the beer wastewater and was evidenced by the accumulation of nitrite ions (40 $mg.L^{-1}$) in the effluent (Figure 6.12) and a substantial drop in biomass levels (Figure 6.10). Facultative denitrifier under limited carbon source availability began to utilize (*RS*)-MCPP more effectively as a carbon source which was due to its inherently lower biodegradability (e.g. propyl side chain only).

The recovery of COD removal efficiency shown by the system when beer wastewater was re-introduced into the influent (COD/N-NO_3^{-1} ratios of 0.2) was almost immediate (Figure 6.2). At the same time, nitrate removal efficiency increased back to 65% within 7 days of the beer wastewater being re-introduced

to the influent. However, due to unintentional fluctuations in feed, nitrate concentration soon began to decline gradually at the COD/N-NO_3^{-1} ratios of 0.2 over 17 days between day 403 and day 420.

The absence of nitrite in the effluent (Figure 6.12) within COD/N-NO_3^{-1} ratios of 250,8,3 and 1 suggests that conversion of nitrate to nitrite was likely to be the rate-limiting step in denitrification (Fang & Zhou, 1999) and lack of nitrite accumulation in the system was due to sufficient organic matter being available for denitrification (Ruiz et al., 2006).

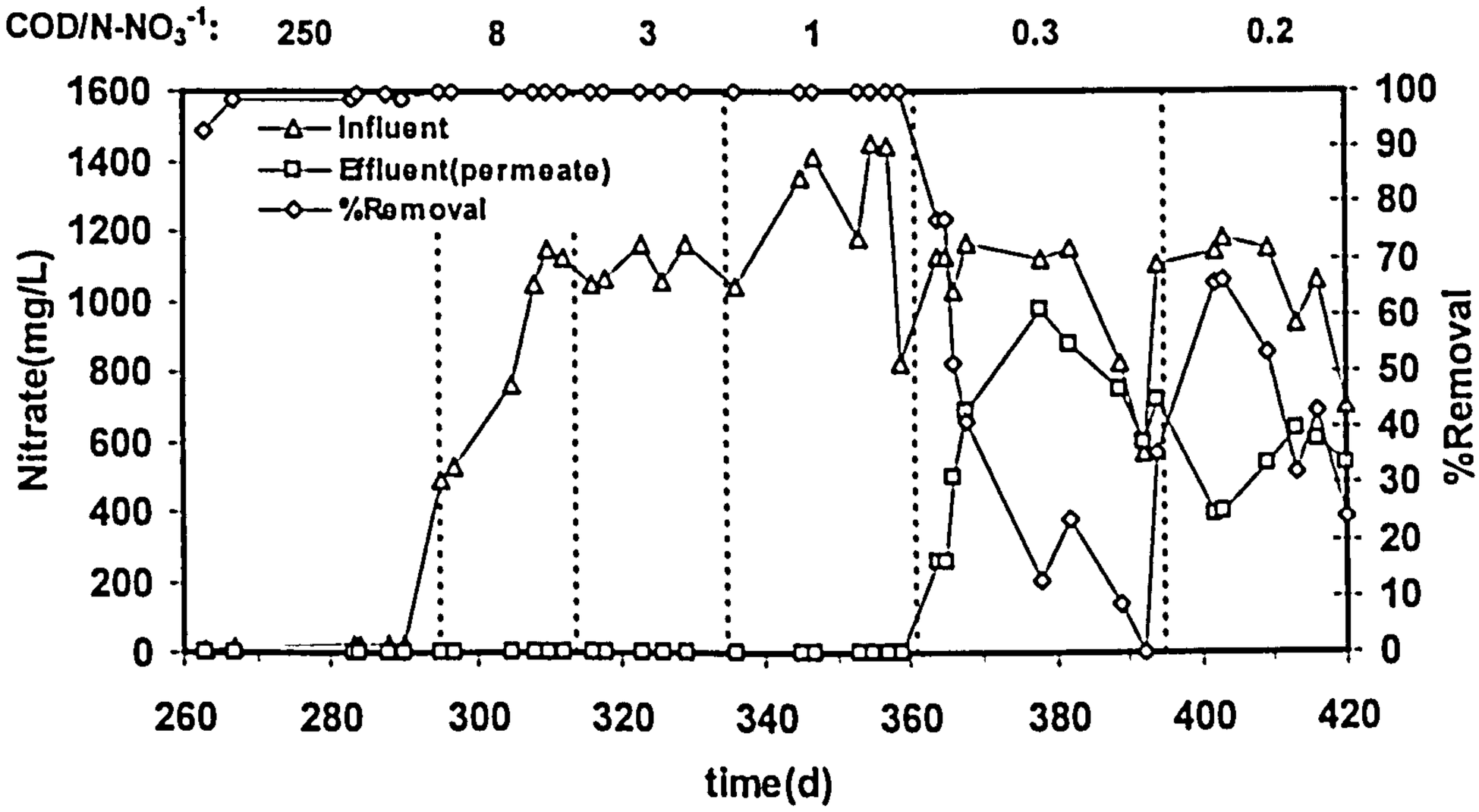


Figure 6.11: Nitrate removal profile of the AMBr under different COD/N-NO_3^{-1} feed ratios.

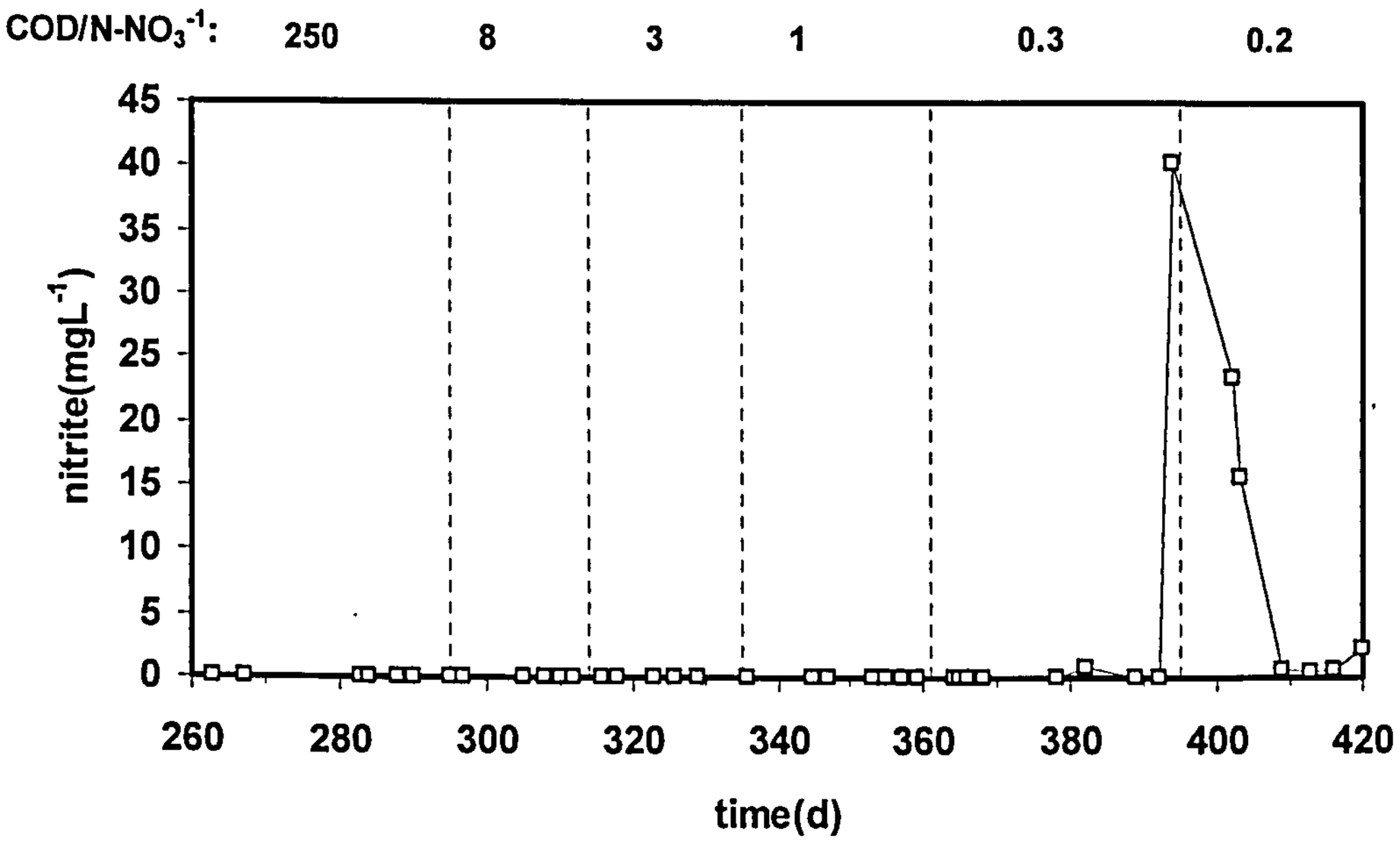


Figure 6.12: Nitrite profile in effluent (permeate) of the AMBr under different COD/N-NO_3^{-1} feed ratios.

6.4 CONCLUSIONS

The following conclusions can be drawn from this study:

- methanogenesis and denitrification can be achieved simultaneously in a single reactor unit, and denitrification in AMBr was strongly dependent on the COD/N-NO_3^{-1} ratio;

- low production of hydrogen assumed during periods when denitrification was stimulated would create a favourable environment for syntrophic acetogenic bacteria to produce substrates for methanogens; however, ammonium inhibition, different substrate affinity or nitrate inhibition resulted in denitrification predominating over methanogenesis;

- (*RS*)-MCPP specific utilisation rate was inversely proportional to the COD/N-NO_3^{-1} ratio;

- (*RS*)-MCPP removal efficiency was affected by factors such as long acclimatization, addition of nitrate as an electron acceptor, and selective pressure all potentially elevating the number of bacteria capable of biodegrading of (*RS*)-MCPP;

- low biodegradation of (*RS*)-MCPP under anaerobic conditions in Phase I with improved biodegradation in Phase II using nitrate as an alternative electron acceptor suggests that the facultative microrganisms were present in the system but were either being inhibited by the strict anaerobic conditions in Phase I or preferentially higher redox conditions in Phase II stimulated a specific enzyme responsible for (*RS*)-MCPP biodegradation;

- at COD/N-NO_3^{-1} ratios 8 and 3, the AMBr was capable of removing 18% (*RS*)-MCPP with a specific utilisation rate of 12 $\mu g.mgVSS^{-1}.d^{-1}$ with concomitant methane production of 2 - 6 $L.d^{-1}$. This indicates that the AMBr is an efficient treatment process not only in removing pollutants such as (*RS*)-MCPP in the wastewater but show high potential for removing nitrate (preventing eutrophication) and producing valuable biogas (methane).

CHAPTER SEVEN

THE EFFECT OF (*RS*)-MCPP DEGRADATION UNDER ANOXIC CONDITIONS (SULFATE REDUCING BACTERIA)

7.1 INTRODUCTION

Alternative terminal electron acceptors, such as sulphate may be present at significant concentrations in the sources where (*RS*)-MCPP is frequently detected such as municipal wastewater, surface water, landfill leachate and groundwater (Bucheli et al., 1998; Petrovic & Larsson-Kovach, 1996; Zipper, et al., 1998; Fletcher et al., 2004). These will have a bearing on the redox conditions and affect the composition of the microbial populations in any reactor system treating these sources, both of which are likely to have an impact on the fate of (*RS*)-MCPP during treatment.

In the anaerobic treatment of sulphate containing wastewater, sulphate reducing bacteria (SRB) use sulphate or sulphur (and other sulphur oxyanions) as a terminal electron acceptors to oxidize a wide range of organic and inorganic compounds (Hansen, 1993). Furthermore, competition exists between SRB and methane production bacteria (MPB) for available reduced carbon compounds such as acetate has been demonstrated elsewhere (O'Flaherty et al., 1998; Omil et al., 1998; Battacharaya et al., 1995; Barber & Stuckey, 2000), and may affect substrate availability and therefore the pathway and extent (*RS*)-MCPP degradation.

In this study, the potential of sulphate as a terminal electron acceptor to oxidize (*RS*)-MCPP will be assessed by investigating the performance of AMBr under different COD to sulphate ratios in the presence of (*RS*)-MCPP.

7.2 METHODS

This experimental study (Phase III) was a continuation of the nitrate-reducing condition in Chapter 6 and investigated sulphate as an alternative terminal electron acceptor. The COD:sulphate ratio was used as a key parameter in the partitioning of (*RS*)-MCPP degradation methane-producing system and sulphate-reducing system.

The reactor was operated at COD:SO_4^{-2} ratios of 2, 0.4 and 0.2 (Table 7.1) for 117 d which was achieved by adding an appropriate quantity of potassium sulphate to the feed regime. Unlike Phase II, the AMBr was operated at a constant OLR of 0.07(±0.01) $kgCOD.m^{-3}.d^{-1}$ by maintaining a hydraulic retention time (HRT) of 3.3 d and COD concentration from beer wastewater of 200 $mgCODL^{-1}$. Throughout the study, pH, COD, VFA, biogas production, (*RS*)-MCPP degradation, MLVSS and MLSS, sulphate reduction were measured according to standard methods (Section 4.6, Chapter 4).

Table 7.1: Summary of the AMBr operating conditions in Phase III.

OLR[a]	COD/ SO_4^{-2}	Day	HRT (d)	(*RS*)-MCPP ($mg.L^{-1}$)	Influent COD[b,c]	Sulphate ($mg.L^{-1}$)
0.07	2	422-468	3.3	50	200	100
0.07	0.4	469-512	3.3	50	200	500
0.07	0.2	513-539	3.3	50	200	1000

[a]= average value ($kgCOD.m^{-3}.d^{-1}$), [b]= $mg.L^{-1}$, [c]= average COD

7.3 RESULTS & DISCUSSION

7.3.1 pH

Initially pH dropped over 20 d from pH 8.3 to pH 7.4 (day 443) due to lower hydroxyl ion (OH^{-1}) being produced in the system (since nitrate was no longer added in the influent) and thus decreased the system pH. Thereafter, no significant changes in pH levels were observed (pH levels were between pH 7.5 to pH 7.8) even after the COD:SO_4^{-2} ratio was decreased to from 2 to 0.4 and 0.2. Above neutral pH in the system (alkaline) suggest that bicarbonate ions (HCO_3^{2-}) could have been accumulate from the by-product of sulphate reduction reactions.

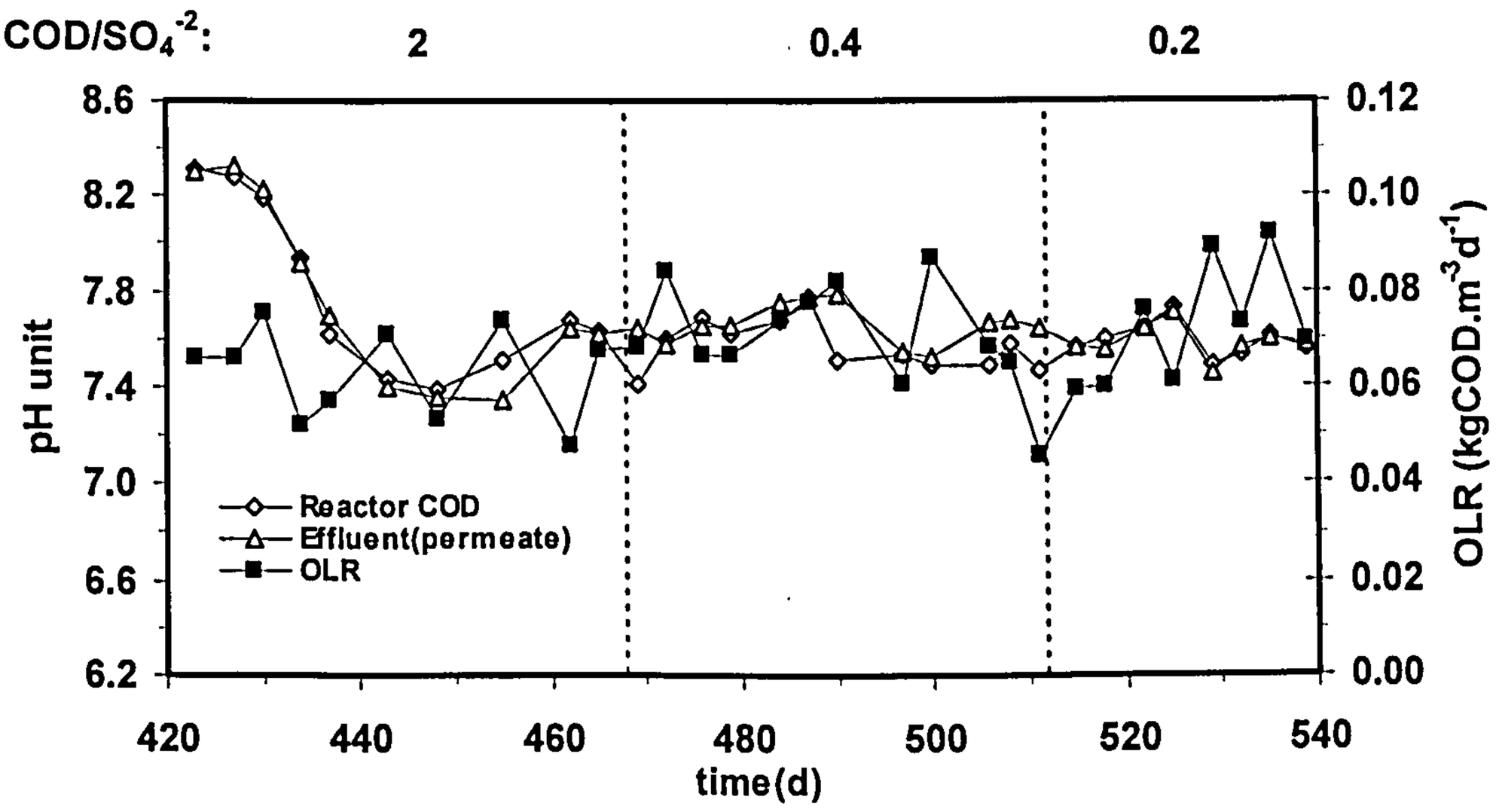


Figure 7.1: pH of the AMBr under different COD/SO_4^{-2} feed ratio.

7.3.2 COD Removal

The total COD removal efficiency declined gradually over time (Figure 7.2) but the contribution from sulphate reduction increased over the same period and showed that sulphate reduction, or its products (sulphide) had a detrimental effect on COD removal efficiency via methanogenesis. There are several possible reasons why COD removal via methanogenesis decreased, such as precipitation of Fe by sulphide ions (which would inhibit methane production), methanogens being out competed by sulphidogens (higher substrate affinity) or direct H_2S inhibition of the methanogens (Freese & Stuckey, 2004).

Considering the theoretical stoichiometry, the reduction of 96 g of sulphate requires 64 g of COD for the sulphate to be reduced to 32 g of sulphide. Therefore, knowing the amount of sulphate reduced in the AMBr (Figure 7.8), it is possible to calculate the amount of COD used for sulphate reduction (Figure 7.2). On day 437, 15 d after the start of Phase III (day 422), the COD removed via sulphate reduction was stable at 20(±4.4)%. It should be noted that if all the sulphate added to the reactor was reduced in the system then at a $COD:SO_4^{-2}$ ratio of 2, 70 $mg.L^{-1}$ of COD would have been removed by sulphate reduction; equating to 35% of the total influent COD. From the data presented in Figure 7.2 it can be seen that the maximum amount of COD removed via sulphate reduction is 40 $mg.L^{-1}$, which is around 20% of the total influent COD. This shows that at a $COD:SO_4^{-2}$ ratio of 2, the reactor is performing at approximately 60% of its theoretical maximum COD removal by sulphate reduction.

At COD:SO_4^{-2} ratios of 0.4 and 0.2, the system still removes more than 65% of the total COD, however, it is apparent that up to 60%-80% of the COD removed is via sulphate reduction, hence reducing the quantity of COD available for conversion to methane. The reactor showed intermittent failure in COD removal by sulphate reduction due to two possible factors: sulphide toxicity (by-product of sulphate reduction) and insufficient COD available for the reduction of sulphate. At these ratios (0.4 and 0.2), if all the sulphate in the feed (500 $mg.L^{-1}$ and 1000 $mg.L^{-1}$ respectively) was to be consumed, the maximum amount of COD needed was insufficient (needed 340 $mg.L^{-1}$ and 670 $mg.L^{-1}$, respectively accounting for 170 % and 340 % of the total influent COD respectively).

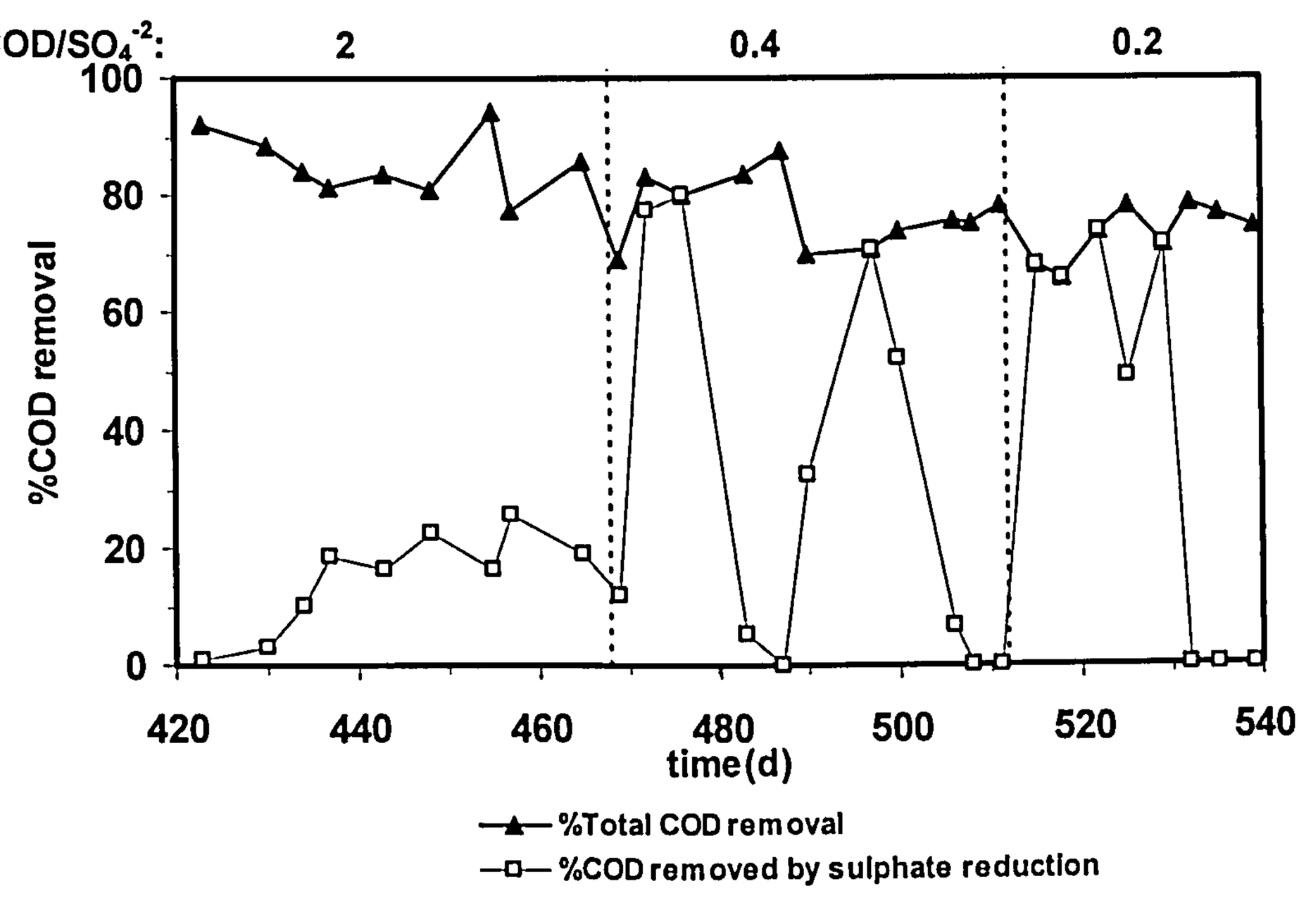


Figure 7.2: COD removal efficiency of the AMBr under different COD/SO_4^{-2} feed ratios.

7.3.3 VFA

The total VFA concentration, which is mainly acetic acid, was low (below 20 $mg.L^{-1}$) throughout Phase III. Analysing some of the essential biochemical reactions involving simple acids might give a clearer view of the basis for good VFA removal in this discussion. Table 7.2 presents a list of primary reactions of anaerobic degradation using simple acids under sulphate and sulphate-free conditions. Accumulation of acetic acid suggests that acetate was not a preferred pathway compared to propionate for SRB because acetate-utilizing SRB are very sensitive to sulphide toxicity i.e. low K_i values (Table 7.2, Reaction 2).

Propionate is a key intermediate in anaerobic digestion and a substrate for all SRB (Chen et al., 2007), was observed only on day 469, 511, 515 and 529 at concentration below 2.5 $mg.L^{-1}$. Maillacheruvu & Parkin (1996) assumed that the predominant reaction was to be incomplete propionate oxidation by sulfidogenic bacteria due to thermodynamic considerations and high K_i values (Table 7.2, Reaction 4). Unlike in methanogenesis, propionate oxidation by SRB is independent of external hydrogen concentrations, and syntrophic growth is not required (Barber & Stuckey, 2000) which further explains significantly improved propionate degradation in the presence of SRB.

Butyrate was not detected throughout Phase III because pH levels in the system were above neutral (pH 7.5 to pH 7.8 – Figure 7.1). McCarty & Mosey (1991) have proposed that butyrate is only produced in significant concentrations as a mechanism to counteract excessively low pH.

Table 7.2: Anaerobic degradation of simple acids under sulphate and sulphate free conditions (Taken from Barber & Stuckey, 2000).

Reaction	ΔG, kJ/mol	K_s mg/L	K_i mg/L
1 $Acetate^- + H_2O \longrightarrow CH_4 + HCO^-_3$	-310	26-300	110
2 $Acetate^- + SO_4^{2-} \longrightarrow HS^- + 2HCO^-_3$	-47.6	14	8
3 Propionate- $+ 2H_2O \longrightarrow Acetate^- + HCO_3^- + H^+ + 2H_2$	+76.1	158	
4 Propionate- $+ 0.75SO_4^{2-} \longrightarrow Acetate^- + HCO_3^- + 0.75HS^+ + 0.25H^+$	-37.7	41	194
5 Propionate- $+ 1.75SO_4^{2-} \longrightarrow 3HCO_3^- + 1.75HS^- + 0.5H^+ + 0.25OH^-$	-106.4	27	25
6 Butyrate- $+ 2H_2O \longrightarrow 2Acetate^- + H^+ + 2H_2$	+48.3		
7 Butyrate- $+ 0.5SO_4^{2-} \longrightarrow 2Acetate^- + 0.5HS^- + 0.5H^+$	-27.8		
8 Butyrate- $+ 2.5SO_4^{2-} \longrightarrow 4HCO_3^- + 2.5HS^- + 0.75H^+ + 0.25OH^-$			
9 4Hydrogen $+ HCO_3^- + H^+ \longrightarrow CH_4 + 4H_2O$	-33.9	13-75	625
10 4Hydrogen $+ HCO_3^- + SO_4^{2-} \longrightarrow HS^- + 4H_2O$	-38.1	8-13	148

K_i = inhibition coefficient, K_s = half saturation constant, ΔG = thermodynamic

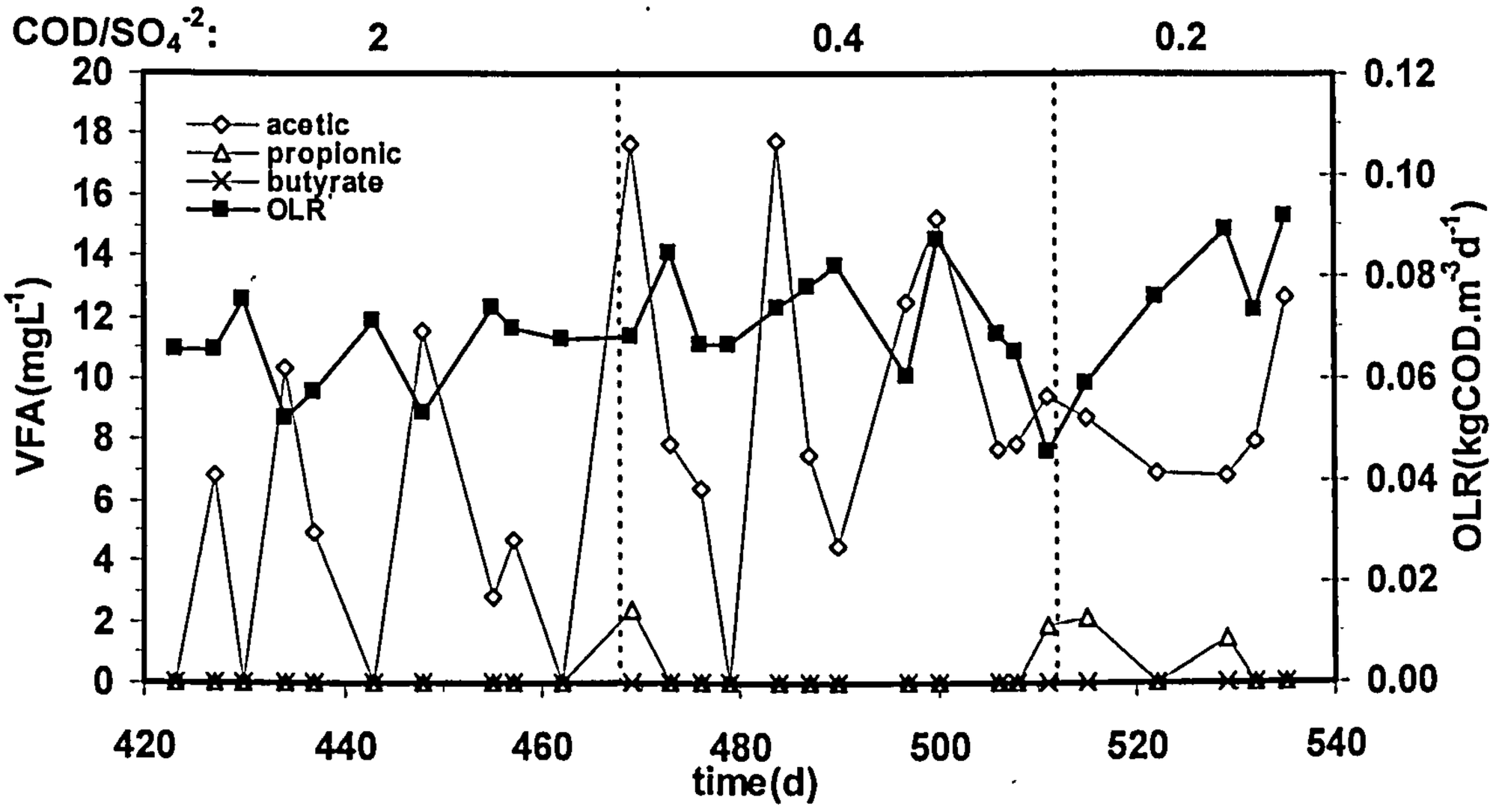


Figure 7.3: Total VFA of the AMBr under different COD/SO_4^{-2} feed ratios.

7.3.4 Biogas

Methane production was 0.01 $L.d^{-1}$ at a COD:SO_4^{-2} ratio of 2. However, at a ratio of 0.4 and 0.2, the methane production ceased to below detection levels. This might be due to several possible reasons as mentioned earlier in Section 7.3.2; such as metal precipitation, directly H_2S inhibition and methanogens being out compete by sulphidogens.

Although metal such as iron, cobalt, nickel and others are essential nutrients to methanogens (Maillacheruvu et al., 1993), high sulphide production from sulphate reduction will result in metal precipitation and could cause metal-nutrient deficiency thus inhibiting methanogenesis.

Koster et al (1986) reported that H_2S inhibited acetoclastic methanogenesis at a concentration of 250 $mg.L^{-1}$. Although no measurement was taken on H_2S and HS^- in the current study, indirect evidence to support H_2S and HS^- accumulation from sulphate reduction by product in the reactor exists (as shown in Table 7.2, Reaction 4). Low propionate concentrations detected in the system showed that Reaction 4 (Table 7.2) was functioning well, hence H_2S and HS^- produced as a by-product would allow metal precipitate formation and the potential inhibition of methanogens.

An appreciation of the competition between SRB and methanogens will provides better understanding of how wastewaters rich in sulphate may be treated anaerobically. SRB have a significantly lower K_s (substrate concentration at

which the reaction rate is half the maximum rate- Reaction 2, 4 and 10, Table 7.2) compared to methanogens (Reaction 1, 2 and 9, Table 7.2) thus the SRB should out compete methanogens for substrates, hence producing less methane.

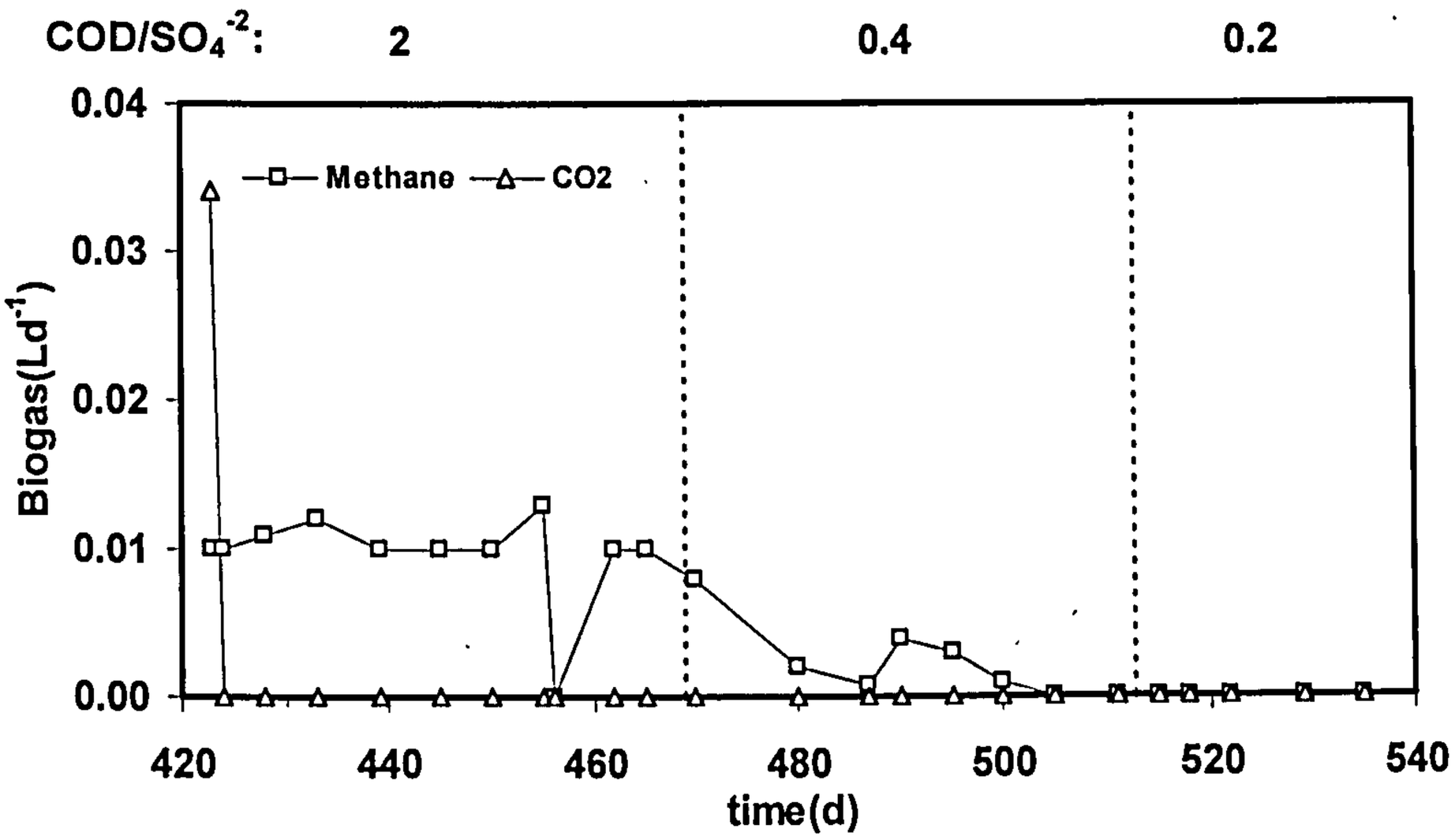


Figure 7.4: Biogas production of the AMBr under different COD/SO_4^{-2} feed ratios.

7.3.5 (*RS*)-MCPP

It was clearly observed that the percentage removal of (*RS*)-MCPP dropped from 46% to 11% and coincided with a rapidly reduction in (*RS*)-MCPP specific utilisation rates from 65.6 to 8.3 $\mu g.mgVSS^{-1}.d^{-1}$ at $COD:SO_4^{-2}$ ratio of 2 (Figure 7.5 and 7.6). Highest (*RS*)-MCPP removal (46%) and SUR (65.6 3 $\mu g.mgVSS^{-1}.d^{-1}$) at day 425 occurred only 3 days after sulphate addition commenced and therefore the actual (8.3 $\mu g.mgVSS^{-1}.d^{-1}$) level observed at this time might be due to involvement of nitrate reducing bacteria in degrading the (*RS*)-MCPP in the biomass. This theory is supported by the reactor pH data (Figure 7.1) which

showed pH 8.2, the optimum pH for denitrification and the COD data (Figure 7.2) which indicates the sulphidogenic route was not the dominant one (percentage COD used for sulphate reduction was only 1%).

At COD:SO_4^{-2} ratios of 0.4 and 0.2, the average (*RS*)-MCPP removal were 15%, 26% with an average (*RS*)-MCPP SUR of 11.4 and 29.6 $\mu g.mgVSS^{-1}.d^{-1}$ respectively. Although the data is limited by the numbers of analyses that were carried out, it appears that some acclimation may have occurred because the average removal and (*RS*)-MCPP SUR at 0.2 ratio (26% and 29.6 $\mu g.mgVSS^{-1}.d^{-1}$) appeared higher than the average removal and (*RS*)-MCPP SUR (15% and 11.4 $\mu g.mgVSS^{-1}.d^{-1}$) at 0.4 ratio (Figure 7.5 and 7.6).

Effects of COD:SO_4^{-2} ratios on the (*RS*)-MCPP SUR of (*RS*)-MCPP and COD were similar in which the effects were more apparent at lower ratios (Figure 7.6). The OLR was maintained at 0.07 $kgCOD.m^{-3}.d^{-1}$, however, at lower ratios, selective pressure on bacteria to degrade (*RS*)-MCPP due to insufficient COD being available for the reduction of sulphate as discussed in Section 7.3.2 could be another contributing factor along with acclimatisation which accounts for better (*RS*)-MCPP SUR and (*RS*)-MCPP removal efficiency.

It appears that in this microbially active environment, microorganisms for (*RS*)-MCPP degradation are present (Phase II) but either sulphate as an electron acceptor did not produce high redox potential environment as nitrate to stimulate enough specific enzyme to degrade (*RS*)-MCPP or preferentially degrading other

organic compounds were not clear. However, selective pressure, tolerance and acclimation of sulphate reducing bacteria to (*RS*)-MCPP may be possible over long periods as the period for 460 – 540 days shows a gradual and constant improvement in (*RS*)-MCPP degradation efficiency.

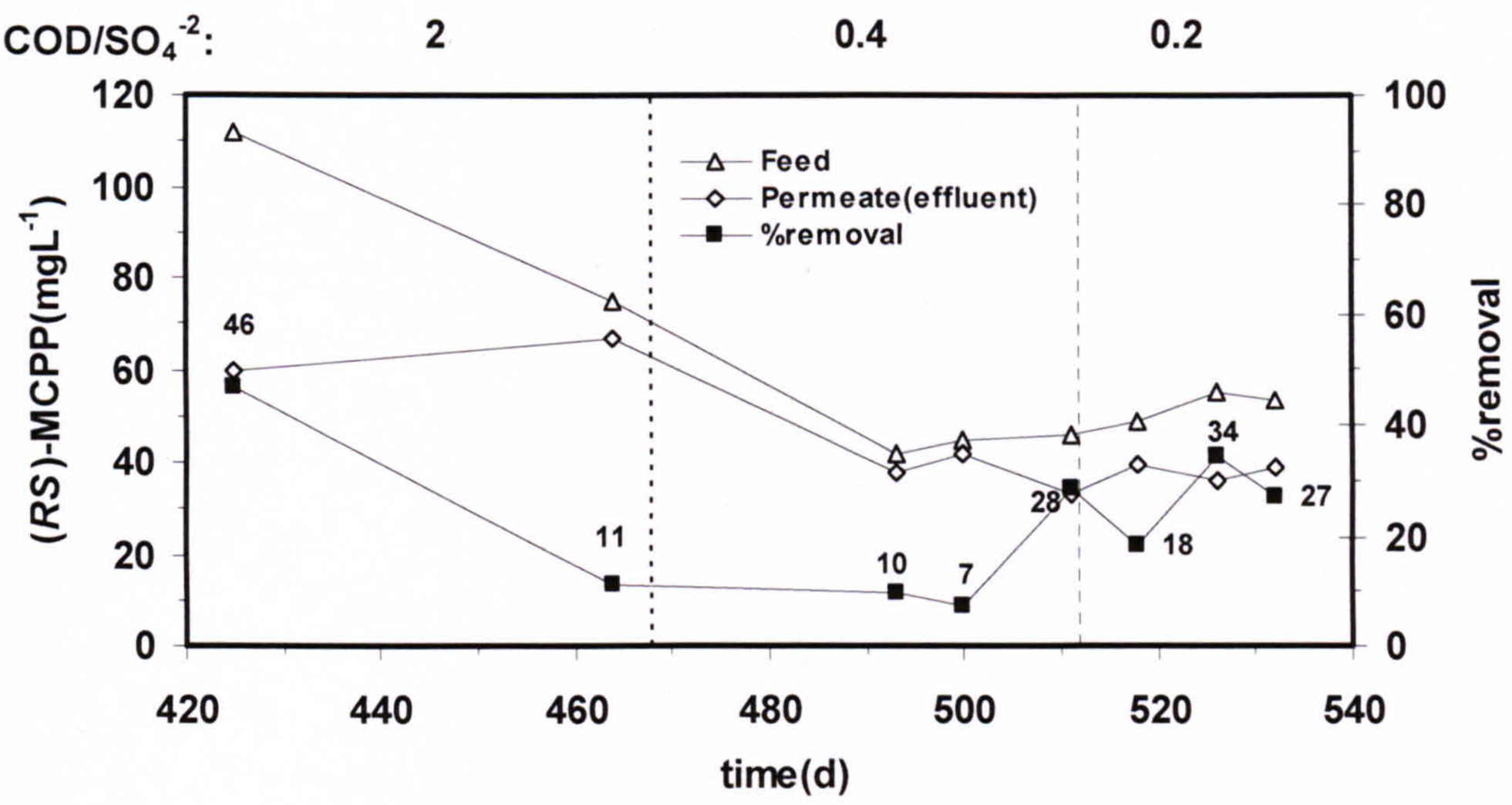


Figure 7.5: (*RS*)-MCPP degradation in the AMBr under different COD/SO_4^{-2} feed ratios.

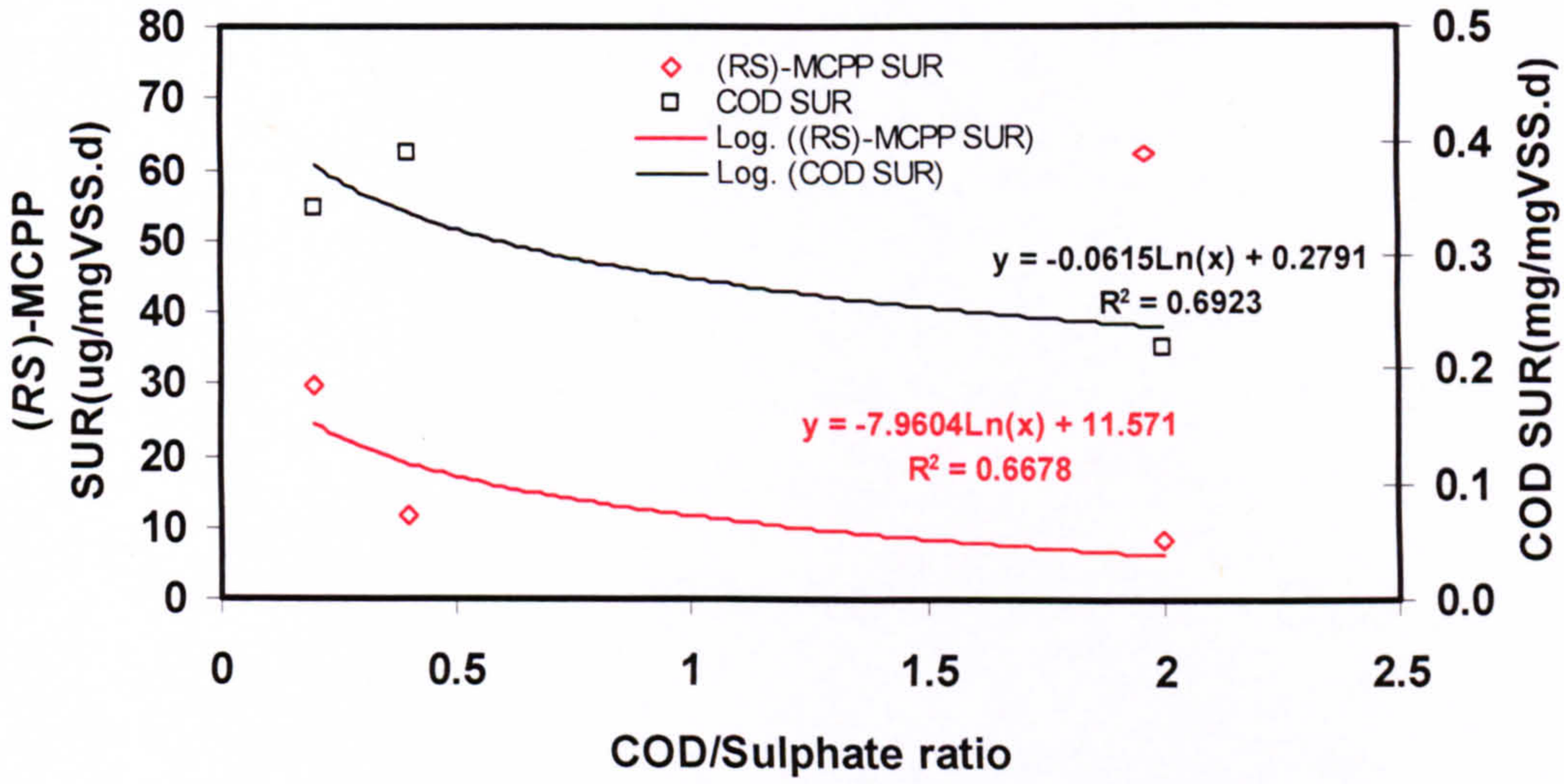


Figure 7.6: Specific COD and (*RS*)-MCPP utilisation rate in the AMBr under different COD/SO_4^{-2} feed ratios.

7.3.6 MLVSS & MLSS

The VSS concentration in the reactor declined gradually at a COD/SO_4^{-2} ratio of 0.4 from approximately 1000 mgL^{-1} to 400 mgL^{-1} before temporarily increasing back to 800 mgL^{-1} then gradually declining to 400 mgL^{-1} at a COD/SO_4^{-2} ratio of 0.2. Mizuno et al., (1994) reported that the growth yield of biomass at each SRT increased with a decrease in the COD/S ratio, and concluded that a large amount of sulphate in the influent can result in a significant increase in bacterial growth. However, this was not observed in the current study and was likely due to insufficient COD being available for sulphate reduction (refer section 7.3.2) and possible sulfide toxicity causing cell numbers (MLVSS) to decline in the system.

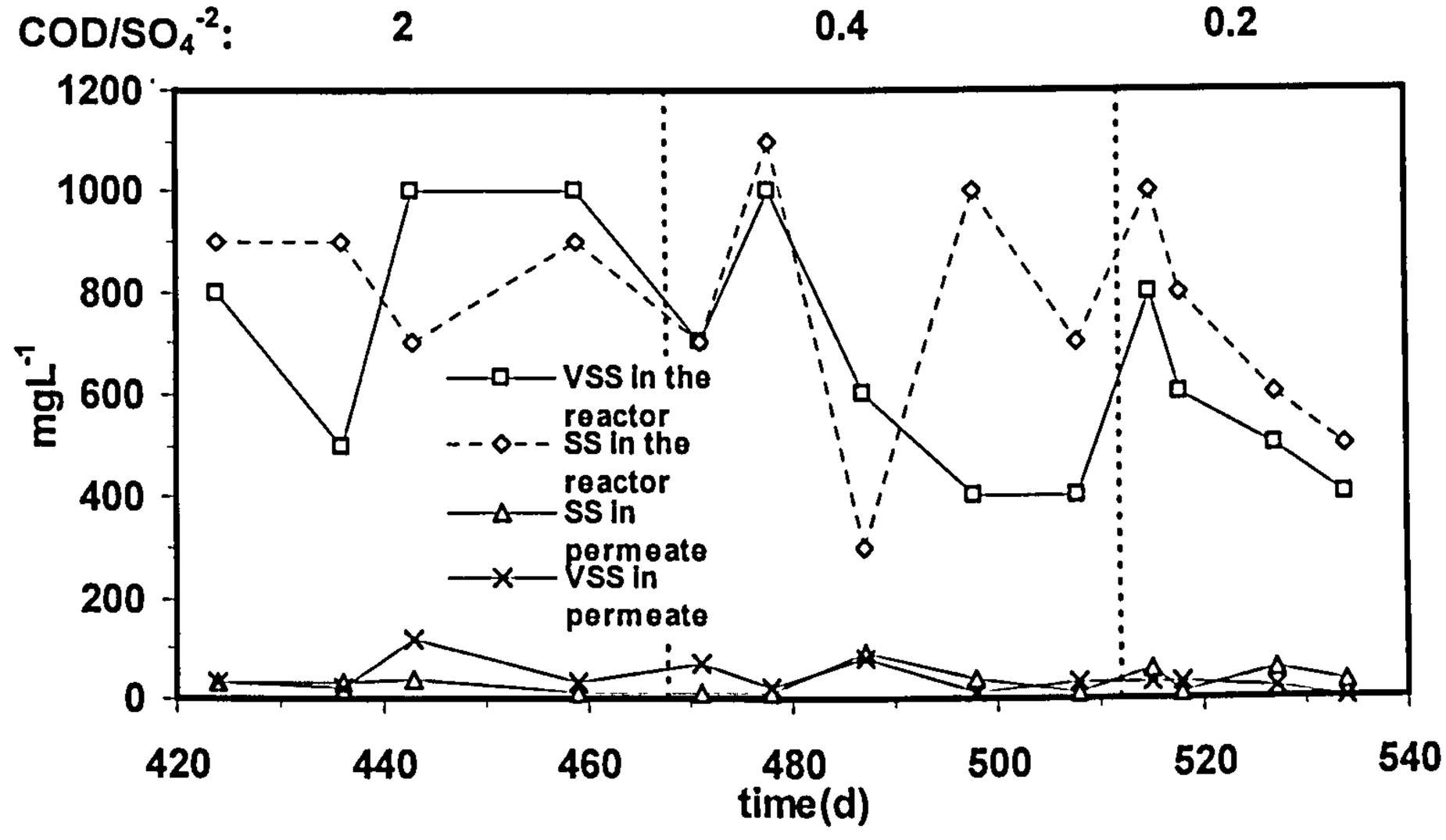


Figure 7.7: SS and VSS of mixed liquor in the AMBr under different COD/SO_4^{-2} feed ratios.

7.3.7 Sulphate reduction

During Phase III the same approach was used as Phase II when the sulphate in the influent beer wastewater was initially kept at a low average concentration of below 70 mgL^{-1} for 35 days. This allowed the sludge to adapt to the new conditions because the seed sludge had no prior exposure to sulphate at these levels. After 15 days of operation, the system reached 76.1% efficiency in sulphate removal. The influent sulphate concentration was increased to 400 mgL^{-1} on day 473, and then to a maximum of 1456 mgL^{-1} on day 529.

A considerable decline and variable efficiency was observed for sulphate removal from day 476 onwards at COD:SO_4^{-2} ratios of 0.4 and 0.2. At this point two factors might have contributed to the intermittent failure in sulphate degradation: insufficient influent COD for sulphate reduction, and substantial sulphide production which could have been toxic to SRB and MPB in the form of dissolved sulphide and undissociated H_2S concentration. Since no measurements were taken for sulphide production during this experiment, it was assumed that higher sulphide accumulation had occurred at COD:SO_4^{-2} ratios of 0.4 and 0.2 compared to COD:SO_4^{-2} ratios of 2 on the basis of comparative reduction in sulphate concentration.

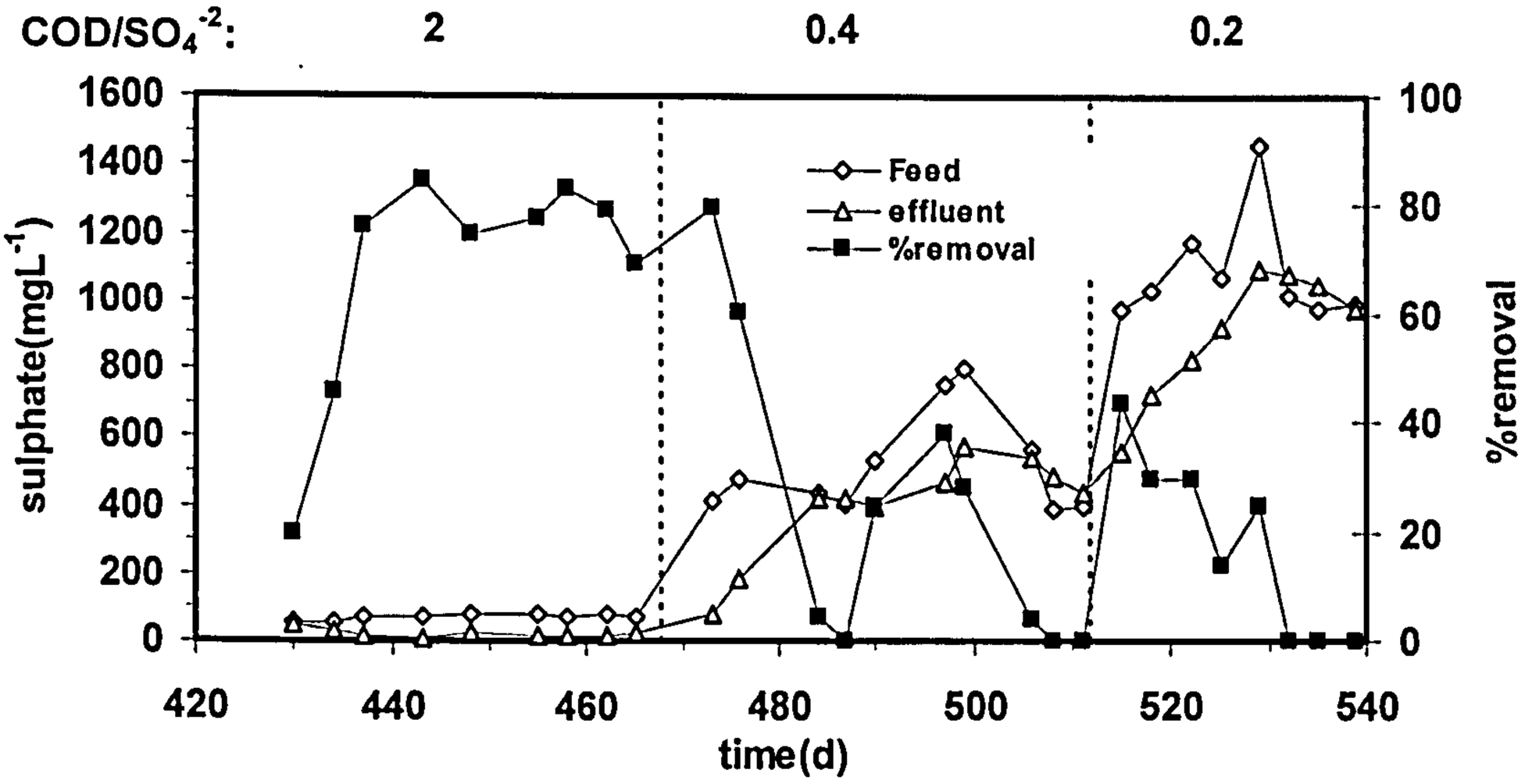


Figure 7.8: Sulphate removal profile of the AMBr under different COD/SO_4^{-2} feed ratios.

7.4 CONCLUSION

The following conclusions can be drawn from this study:

- the methanogens were rendered inactive at $COD:SO_4^{-2}$ ratios of 2 and below and results showed that SRB played a key role in the degradation of COD in the AMBr when sulphate was present;

- appreciation of sulphide toxicity and other factors controlling competition between SRB and other anaerobic trophic groups, suggests it may be possible in the future to treat wastewater containing very low $COD:SO_4^{-2}$ ratios under entirely sulphidogenic conditions whilst maintaining (*RS*)-MCPP removal and to manipulate the operating conditions with intermediate $COD:SO_4^{-2}$ ratios to enhance methanogenesis;

- acclimatisation and selective pressure on bacteria that degrade (*RS*)-MCPP might have an influence on gradual improvement in (*RS*)-MCPP removal efficiency and specific utilisation rate with time;
- from the perspective of (*RS*)-MCPP degradation and specific utilisation rates, the sulphate reducing conditions (Phase III) were considerably less efficient than the nitrate reducing (denitrifying) conditions investigated in Phase II when the (*RS*)-MCPP removal efficiency and (*RS*)-MCPP SUR dropped rapidly during the early part of Phase III.

CHAPTER EIGHT

EFFECT OF HRT ON (*RS*)-MCPP DEGRADATION UNDER METHANOGENIC CONDITIONS

8.1 INTRODUCTION

One of the limitations of of anaerobic treatment (and other biological treatment) processes, i.e. the provision of long retention time (SRT) can be overcome by the introduction of membrane unit which permits SRT and hydraulic retention time (HRT) to be varied independently. This allows improved process stability to be achieved by providing a long SRT which is not dependent on wastewater flows (HRT).

Although the performance of the AMBr configuration has been reported to be relatively insensitive to HRT (Stephenson et al., 2000), changes in HRT by regulating the wastewater flow might have an impact on the contact time between (*RS*)-MCPP (a very soluble molecule) and mixed anaerobic microbial community and be likely to affect the degree of degradation and (*RS*)-MCPP SUR.

In Phase IV of this research, the effects of different HRT on (*RS*)-MCPP utilisation were investigated by decreasing the feed flow rate. Consequently, this led to different OLR being established, and therefore two variables (HRT and OLR) were changed simultaneously whilst investigating (*RS*)-MCPP SUR. This meant that only limited interpretation could be made regarding which of these two variables was the major factor influencing (*RS*)-MCPP degradation, but still

allowed conditions to be identified that provided improved (*RS*)-MCPP. Future experiments should be conducted which disengage these two variables.

Phase V was a repetition work carried out on the Phase IV experiments to verify that (*RS*)-MCPP degradation had not been influenced by trace levels of oxygen headspace of the effluent tank when part of the effluent (permeate) was recycled through the reactor to maintain constant HRT of the process. Thus the objective of this study was to examine the effect of HRT (by decreasing the OLR) on (*RS*)-MCPP utilisation rate at three different HRT and to investigate the robustness and stability of AMBr in response to hydraulic shock loads.

8.2 METHODS

During Phase IV and V, (*RS*)-MCPP concentration in the reactor feed was 50 mgL^{-1}. Three different HRT were investigated; 3.4, 6.8, and 16.9 d in Phase IV, and these were achieved by decreasing the flow rate which gave corresponding OLR of 0.47 $kgCOD.m^{-3}d^{-1}$ to 0.13 $kgCOD.m^{3}d^{-1}$ (Table 8.1). The robustness and stability of AMBr was investigated in response to hydraulic shock loads on the biomass imposed by increasing the flow rate to 20 times higher than that when HRT was at 16.9 d (i.e. HRT was 0.8 d) and maintaining this flow over a period of three weeks before being returned to 3.4 d to investigate the recovery of the biomass.

In Phase V, the reactor was operated at the same operating condition (Table 8.2) as in the previous phase (Phase IV). However, in order to investigate the effect

of trace oxygen in the headspace of the effluent tank towards system performance in Phase IV (HRT 16.9 d), when part of the effluent (permeate) had been recycled into the reactor, the effluent tank was made strictly anaerobic by providing a constant overpressure with nitrogen gas and a manometer seal. The investigation was started at HRT 3.4 d to give the same operating conditions as the start of Phase IV. Once the system has reached steady state conditions, HRT was increased to 16.9 d by operating at the lowest influent flow from Phase IV resulting in a rapid recycle flow of permeate back into the reactor from the effluent tank. The diagram of the effluent tank N_2 overpressure seal (manometer) is shown in Figure 8.1.

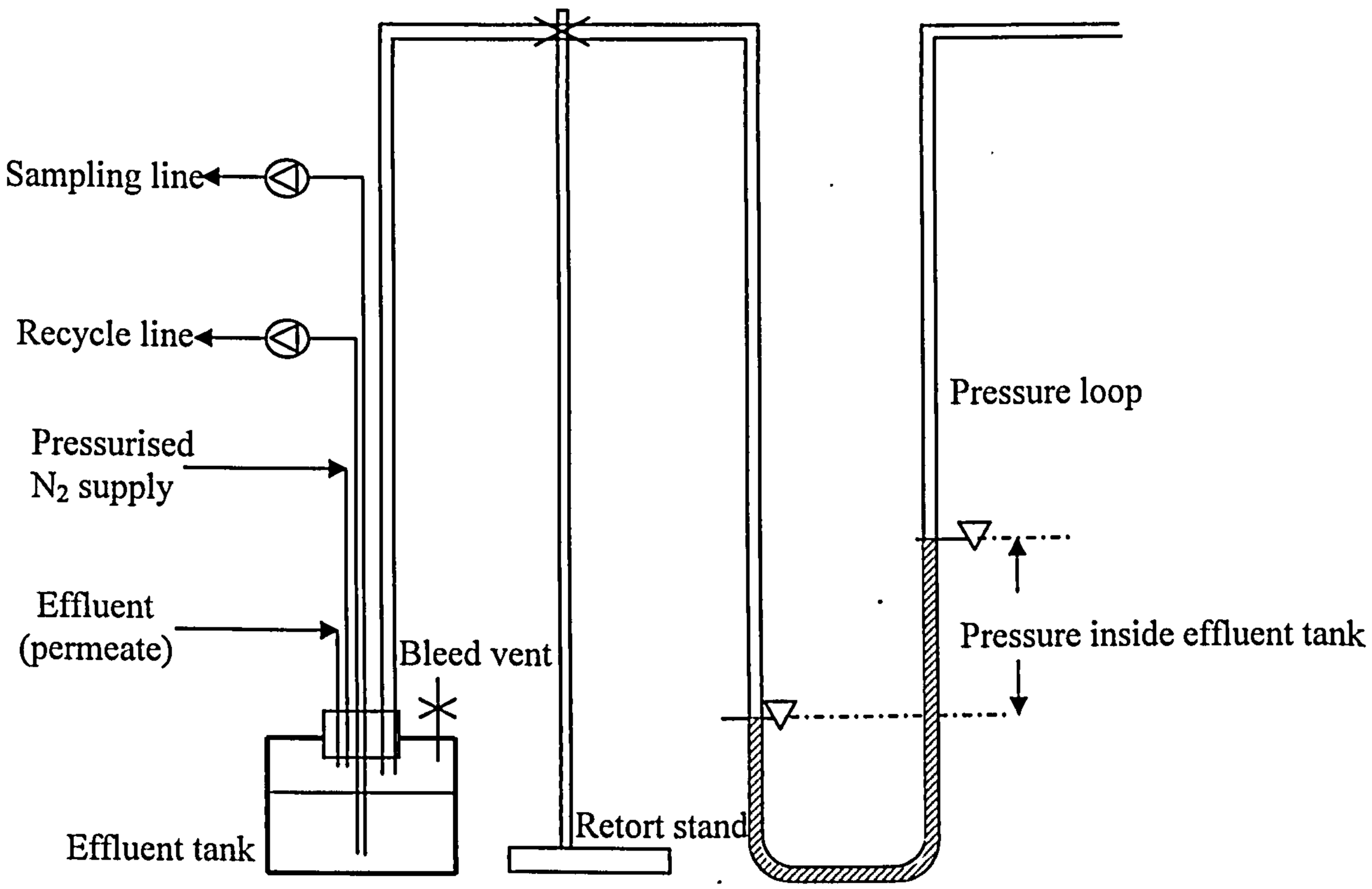


Figure 8.1: Schematic diagram of the effluent tank N_2 over-pressured seal used in Phase V.

Table 8.1: Summary of reactor operating conditions of the AMBr system during the study of HRT on (*RS*)-MCPP degradation under methanogenic condition in Phase IV.

Day	HRT(d)	(*RS*)-MCPP (mgL^{-1})	OLR[a]	Influent COD[b] (mgL^{-1})
542-569	3.4	50	0.47	1570
570-598	6.8	50	0.21	1420
599-623	16.9	50	0.13	950
624-644	0.8	50	2.22	1900
645-672	3.4	50	0.51	1705

[a]= average value ($kgCOD.m^{-3}.d^{-1}$), [b]=average COD

Table 8.2: Summary of reactor operating conditions of the AMBr system in Phase V.

Day	HRT(d)	(*RS*)-MCPP (mgL^{-1})	OLR[a]	Influent COD[b]
673-692	3.4	50	0.42	1380
693-720	16.9	50	0.07	1170
721-740*	16.9*	50	0.09*	1460

[a]= average value ($kgCOD.m^{-3}.d^{-1}$), [b]=average COD, *nitrogen over-pressured seal.

8.3 RESULTS AND DISCUSSION (Phase IV)

8.3.1 pH

The pH level in the reactor and effluent (permeate) followed a similar pattern throughout Phase IV. The pH levels were generally stable (pH 6.9-7.2) at HRT of 3.4, 6.8 and 16.9 d, corresponding to OLR of 0.47, 0.21 and 0.13 $kgCOD.m^{-3}.d^{-1}$, respectively, showing a slight pH increase after each new HRT condition was introduced (Figure 8.2). Salminen and Rintala (2002) also observed four different pH levels of digested material; pH 6.2 (±0.1), 6.9 (±0.1), 7.4 (±0) and 7.5 (±0.1) in four identical stirred anaerobic digesters treating poultry slaughterhouse wastewater operating at HRT of 13, 25, 50 and 100 d.

However, at a reactor HRT of 0.8 d (when the reactor received a shock OLR loading at 2.30 $kgCOD.m^{-3}.d^{-1}$), the pH dropped rapidly to pH 5.9. As a result of

the high concentration of VFA's that resulted from an imbalance between VFA production by acidogens and VFA utilization by the methanogens, net VFA production decreased the pH accordingly in both the reactor and effluent (permeate). The pH levels then eventually stabilised at pH 5.1. However, when the reactor HRT was increased back to 3.4 d, the pH of reactor and permeate recovered rapidly (10 days) to around 6.8, a value that is almost identical to that (pH 6.9) observed previously at HRT 3.4 d.

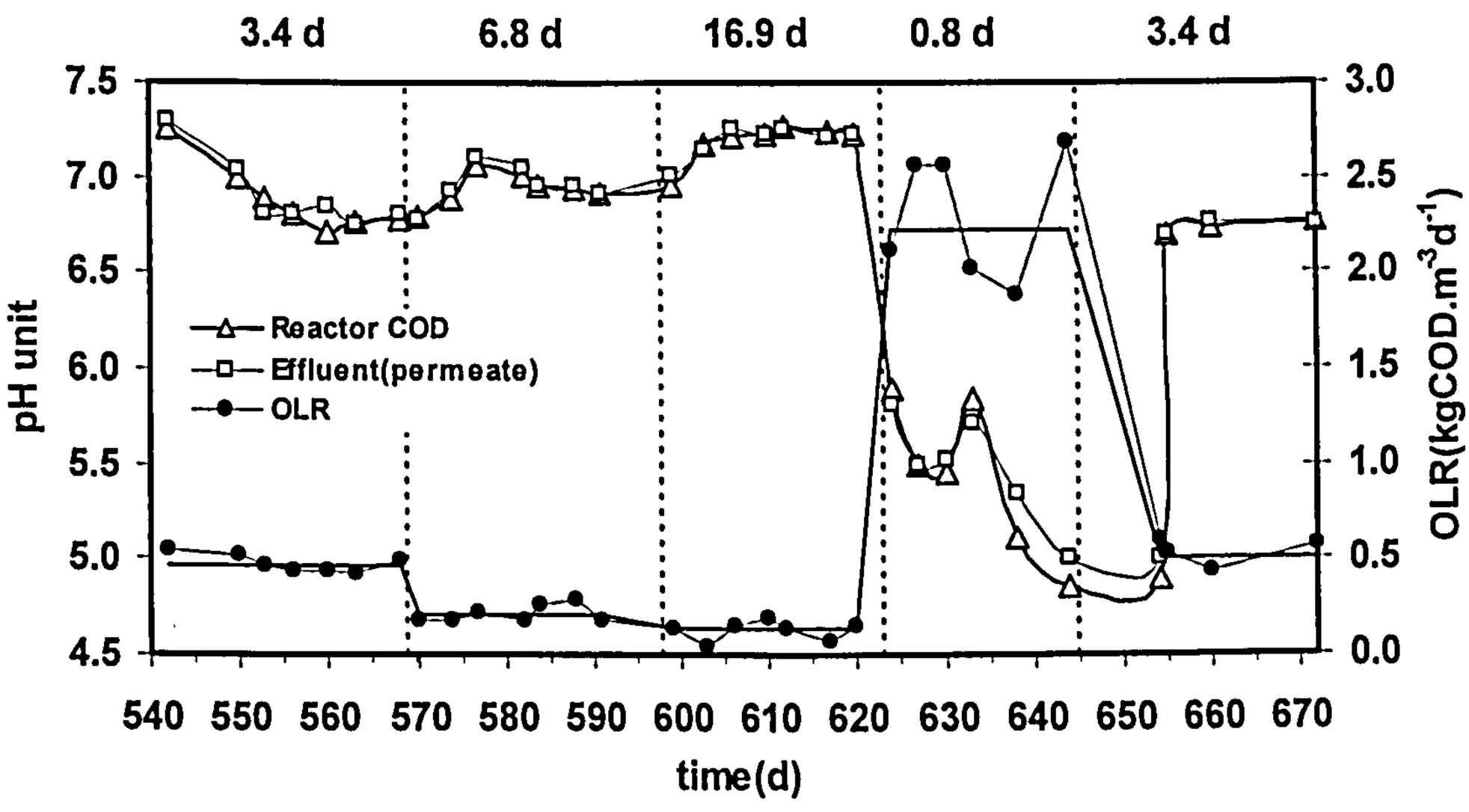


Figure 8.2: pH profile of the AMBr at different HRT.

8.3.2 COD Removal

The average soluble COD removal efficiency was 91.4 (±3.5) %, 96.9 (±1.3) %, and 94.4 (±1.5) %, for HRT 3.4, 6.8 and 16.9 d respectively. However, when the hydraulic shock load (HRT 0.8 d, 2.30 kgCOD.m^{-3}.d^{-1}) was imposed, the soluble COD removal efficiency declined rapidly to 26 % within 6 days before

recovering back and stabilising after a further 14 days to a value of 60 % (Figure 8.3). When the reactor HRT was increased back to 3.4 days (by lowering the flow rate), the total COD removal efficiency recovered quickly (within 2 days) to an average of 95 (±0.7) % despite having been exposed previously to the hydraulic shock load conditions for 21 days. This confirms that any adverse effects on the critical methanogenic population imposed by the acidic conditions at 0.8 d HRT were reversible, allowing almost immediate recovery of activity when the balance between acidogenesis and methanogenesis was restored at HRT 3.4 d. Grobicki and Stuckey (1990) also noticed quick recoveries (within 24 h) of COD removal efficiency (to 96 %) in an anaerobic baffled reactor when soluble COD removal efficiency had previously declined to 20 % - 30 % during a 3 hours shock load period imposed by changing HRT from 20 hours (OLR = 4.8 $kgCOD.m^{-3}.d^{-1}$) to 10 h HRT (OLR = 96 $kgCOD.m^{-3}.d^{-1}$).

The COD peaks observed in the reactor before and after hydraulic shock load, were clustered around a value of 600 $mg.L^{-1}$ but increased sharply to approximately 2000 $mg.L^{-1}$ during hydraulic shock load event. This observation can be explained by considering the implications of SMP production. SMP can be classified into two groups: utilization associated products (UAP) and biomass associated products (BAP), furthermore, substrate concentration significantly affects the quantity of SMP production, more SMP being produced at higher feed concentrations (Schiener et al., 1998). In the current study it was found that as the HRT decreased, the OLR increased and more UAP were produced. These increased rates of UAP production exceeded the rate of UAP degradation,

leading to increased SMP concentrations being present the reactor during periods of hydraulic shock loading.

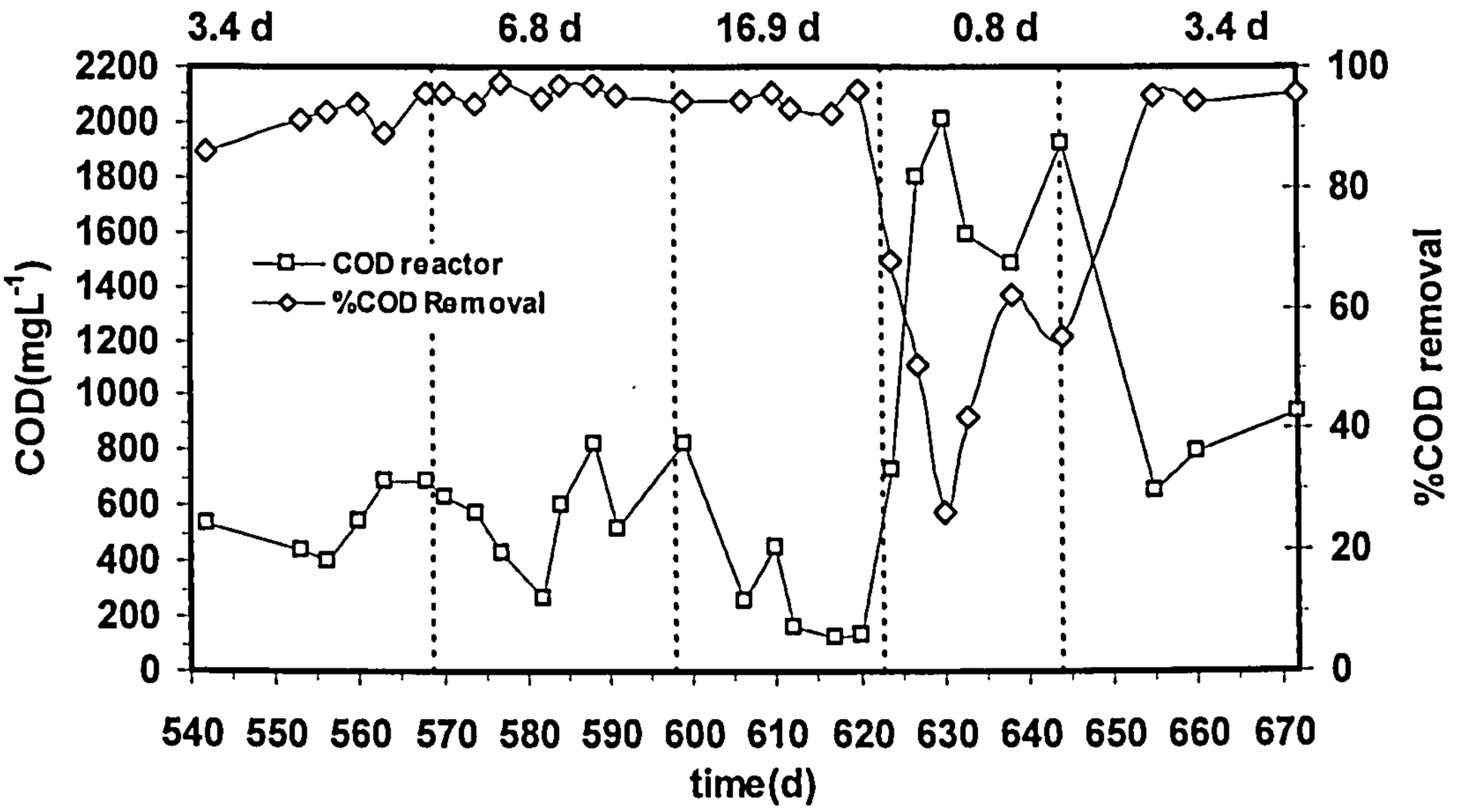


Figure 8.3: COD removal efficiency and COD reactor of the AMBr at different HRT.

8.3.3 VFA

The total VFA production increased rapidly (increase to 300 %) in response to the period of shock hydraulic load (HRT 0.8 d). Before the shock hydraulic load (HRT 3.4 d, 6.8 d, 16.9 d), the total VFA was generally low (below 20 $mg.L^{-1}$). Furthermore, low concentrations of acetate were detected before shock loads, indicating the dominance of *Methanosaeta* with low K_s (20 $mg.L^{-1}$) or possibly other methanogens with high acetate affinity. High acetate accumulation (Figure 8.4) during shock loads caused a substantial decreased in pH, and this may have stimulated butyrate production as a relatively high concentration of butyric acid (up to 110 $mg.L^{-1}$) was detected during the shock load period. It is generally accepted that butyrate is produced under stressed conditions to counteract

excessively low pH (McCarty & Mosey, 1991), and Grobicki and Stuckey (1991) have proposed that since butyrate is not a substrate for methanogens, it also acts as an intermediate reserve for acetate when it is present at high levels, and can be converted back to acetate when acetate concentrations begin to decline.

There was no propionate detected in the reactor at HRT 3.4, 6.8 and 16.9 d which indicates a well functioning anaerobic process (Speece, 1996) but propionate was detected during the 0.8 d HRT; hydraulic shock loads (Figure 8.4). High propionate suggests an unfavourable environment for syntrophic obligate hydrogen producing acetogens which require low hydrogen levels. Propionate was detected only on the first day of Phase IV at HRT of 3.4 d (day 542) at concentration of 1.45 $mg.L^{-1}$ but soon declined to below detection levels and was detected again at HRT 16.9 d (day 606) at a concentration of 1.66 $mg.L^{-1}$.

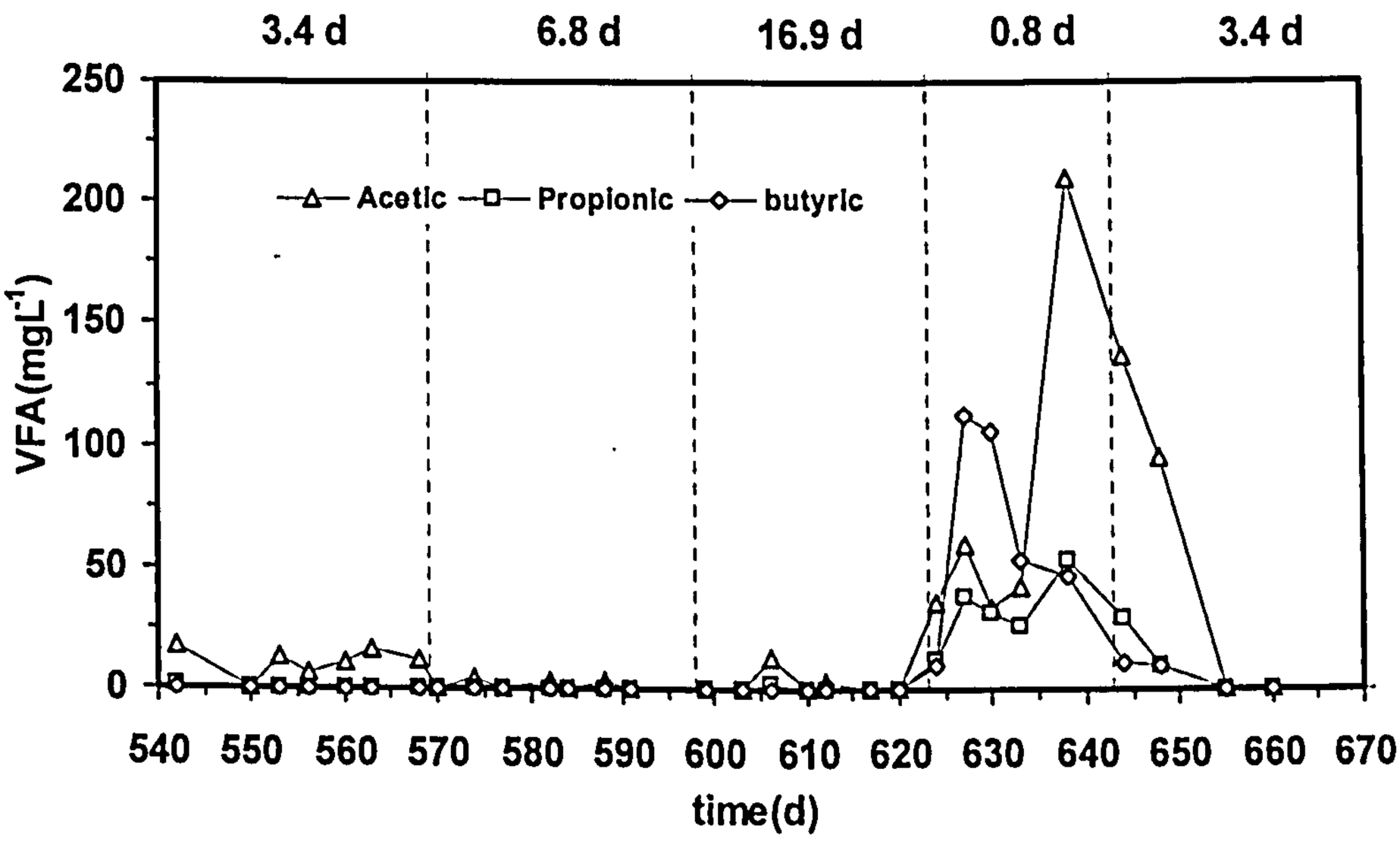


Figure 8.4: VFA concentration in the AMBr throughout Phase IV.

8.3.4 Biogas

It is clear that volumetric methane production reduced in response to longer HRT. Increased HRT (achieved by reducing the flow rate thus reducing OLR) resulted in less COD being available for the anaerobic processes, and consequently methane production in the reactor was 2.5 $L.d^{-1}$, 1.0 $L.d^{-1}$ and 0.09 $L.d^{-1}$ (Figure 8.6) when HRT was set to 3.4 d, 6.8 d and 16.9 d, corresponding to OLR of 0.47, 0.21 and 0.13 $kgCOD.m^{-3}.d^{-1}$, respectively. However, when hydraulic shock load was applied (HRT=0.8 d, OLR=2.30 $kgCOD.m^{-3}.d^{-1}$), methane production increased rapidly up to 6.64 $L.d^{-1}$ within 7 d before stabilising at around 5 $L.d^{-1}$.

Although low acetoclastic activity was observed during shock loading (i.e. acetate accumulated on day 624 - Figure 8.4), relatively high methane production and methane yields during shock loads might be due to enhanced levels of reductive methanogenesis which would have been stimulated by the elevated concentrations of hydrogen occurring during periods of high propionate concentration. Hydrogen-utilising methanogens are known to be capable of removing significant amounts of hydrogen during anaerobic digestion and are capable of responding quickly to elevated hydrogen availability due to their short doubling time of about 6 hours (Mosey, 1983).

The methane yield for HRT 3.4-16.9 d follows the same trend as in methane production. It was observed that the theoretical value of methane yield (0.35 m^3/kgCOD destroyed) is significantly different from the observed methane yield

at HRT 6.8 d and 16.9 d which were only 0.19 and 0.04 m^3/kgCOD destroyed (Figure 8.5), respectively. This deviation could be due to the quantity of methane dissolving in the effluent (methane stripping) being proportionally a much greater fraction of the total methane produced when low OLR was applied to the reactor during times of long HRT. At the lower OLR in the influent, only small volumes of methane were produced and a relatively greater proportion of this dissolved in the liquid phase. Singh, et al., (1996) and Noyola, et al., (1988) experienced 28 - 39 % and more than 50 % loss of methane through solubility in the effluent (rather than appearing in the biogas) when operating at low strength wastewater.

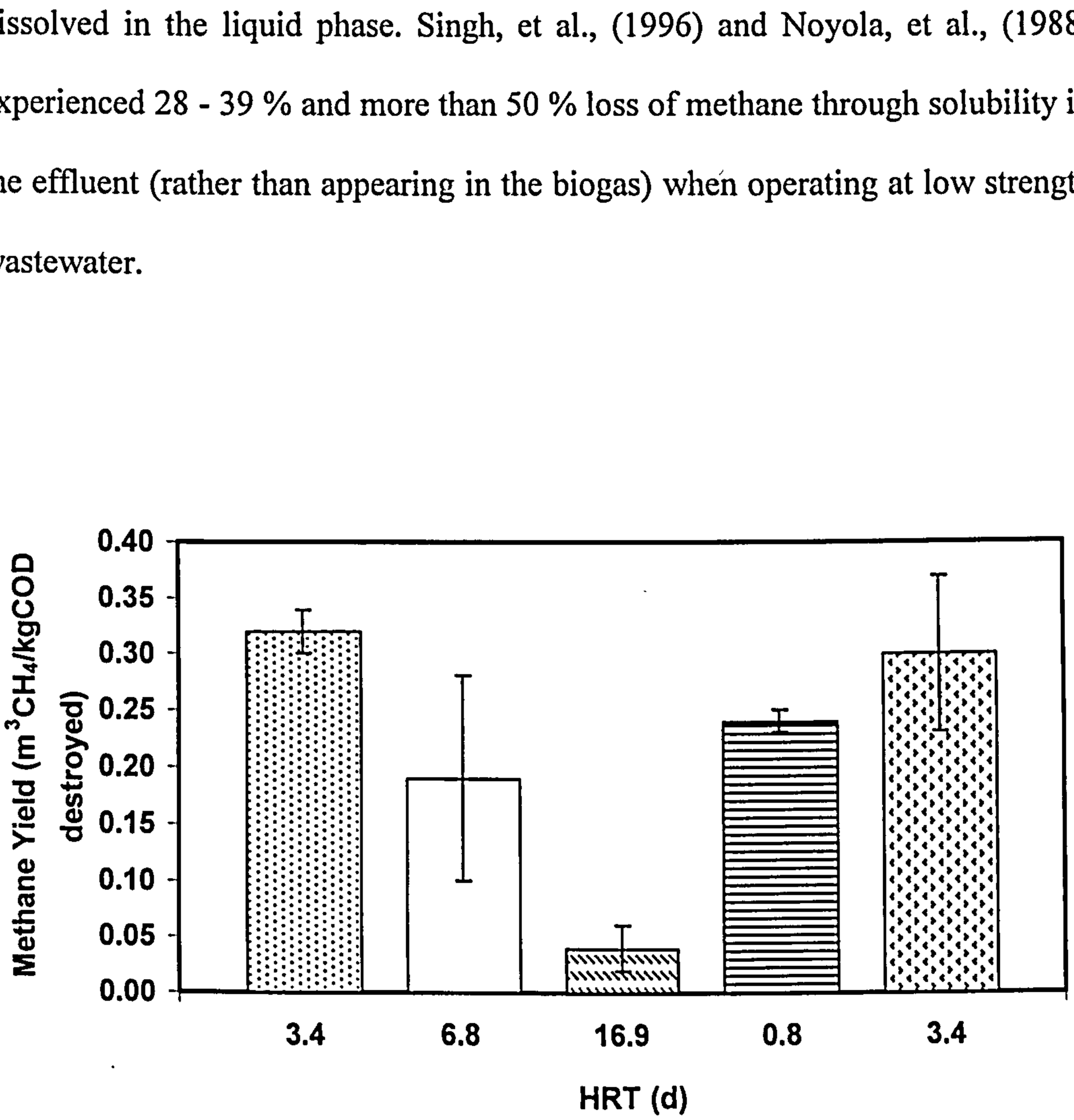


Figure 8.5: Mean methane yield of the AMBr at different HRT.

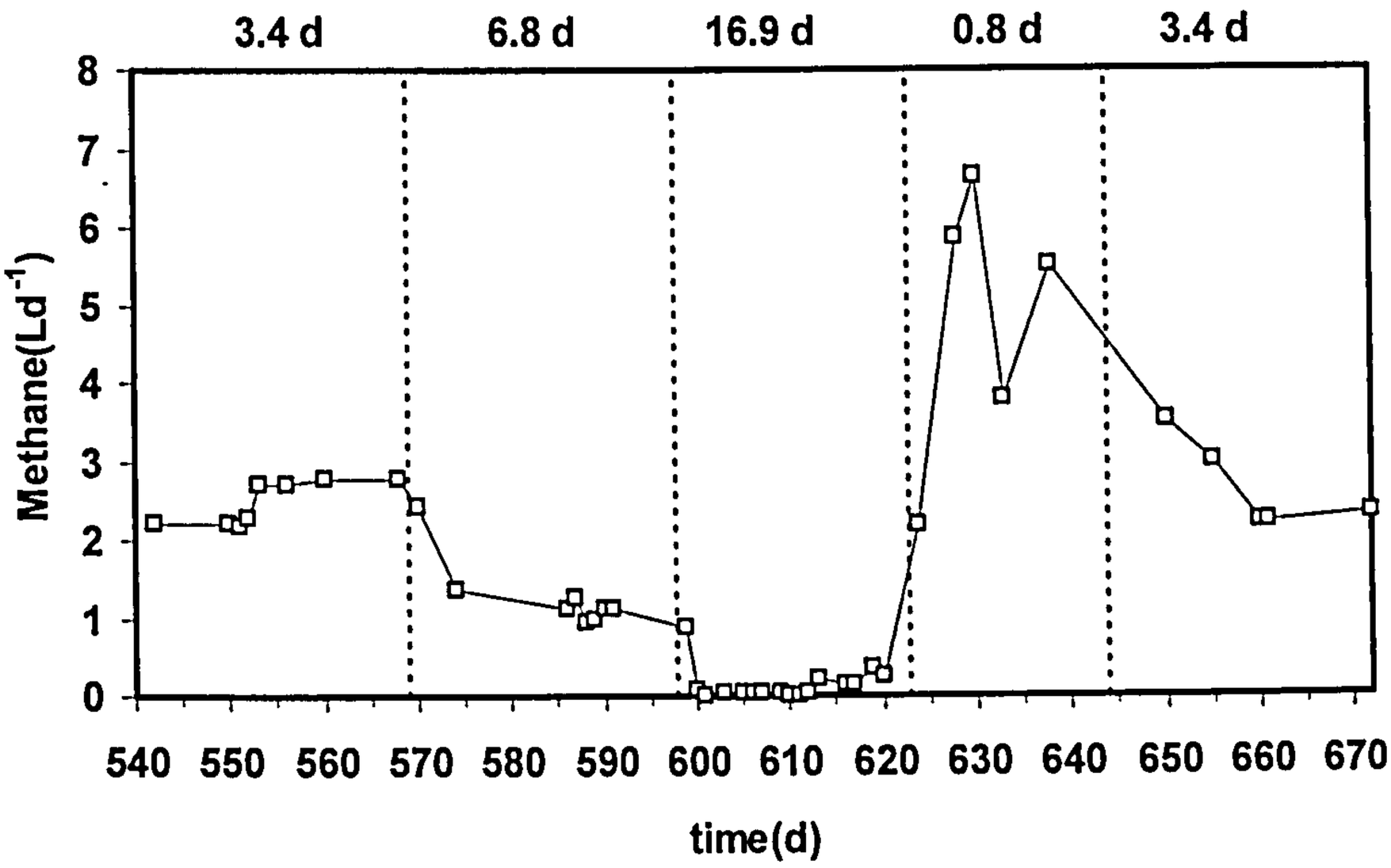


Figure 8.6: Daily methane production of the AMBr at different HRT.

8.3.5 (*RS*)-MCPP Degradation

(*RS*)-MCPP removal efficiency fluctuated from 6-39 % at HRT 3.4 d (OLR 0.47 $kgCOD.m^{-3}.d^{-1}$), however when HRT was increased to 6.8 d and 16.9 d with concomitant decreased in OLR to 0.21 to 0.13 $kgCOD.m^{-3}.d^{-1}$ respectively, the removal efficiency increased to an average of 60 (±1.1) % and 75 (±1.6) % (Figure 8.7). Since there was no decline in sterile (*RS*)-MCPP concentration under stability control tests carried out under similar conditions to the reactor but without biomass and profound decline during reactor HRT and OLR changes from 3.4 d to 16.9 d and 0.47 to 0.13 $kgCOD.m^{-3}.d^{-1}$ respectively, it was concluded that (*RS*)-MCPP degradation was not attribute to an abiotic process. However, operating under lower strength of OLR (higher HRT) which led to effluent been oversaturated with the dissolved gases (as mentioned in section 8.3.4), might have allowed N_2 and O_2 to enter the reactor via permeate recycling

line from the effluent tank, possibly contributing to some aerobic (*RS*)-MCPP degradation. This is discussed below in more detail under Phase V (Section 8.4).

Harrison et al. (2003) has reported that (*S*)-MCPP did not degrade but (*R*)-MCPP was degraded with zero order kinetics at 0.65 mgL^{-1} under nitrate-reducing conditions in anaerobic microcosms, however from the degradation result obtained in the current study during HRT 6.8 d and 16.9 d at OLR of 0.21 and 0.13 $kgCOD.m^{-3}.d^{-1}$ respectively (60 % and 75 % removal efficiency), it was found that not only (*R*)-MCPP but also (*S*)-MCPP was biodegraded under methanogenic conditions since the racemic mixture was 1:1 ratio and failure to degrade (*S*)-MCPP would have resulted in a measurement of 50 % degradation. Furthermore, Harrison et al., (2003) confirmed in their work that no chiral conversion (inversion of (*R*)-MCPP to (*S*)-MCPP) took place when (*R*)-MCPP concentration declined. During hydraulic shock loads, the removal efficiency dropped sharply to only 4 (±0.6) % (average), but the removal efficiency improved to 19 % when the reactor HRT was increased to 3.4 d (OLR 0.47 $kgCOD.m^{-3}.d^{-1}$).

Another interesting observation was that the (*RS*)-MCPP specific utilisation rates (SUR) were dependent on the HRT and OLR (Figure 8.8 and 8.9) and gradually improved from 23 to 83 $\mu g.mgVSS^{-1}.d^{-1}$ as flow rate increased which caused HRT to increase from 3.4 d to 16.9 d with concomitant decreases in OLR from 0.47 to 0.13 $kgCOD.m^{-3}.d^{-1}$. In contrast, COD SUR was relatively more stable and less dependent on HRT and OLR. This suggests that two factors might have

an influence on the AMBr achieving improved (*RS*)-MCPP SUR during Phase IV; namely HRT and OLR.

(*RS*)-MCPP has high solubility in the water (700 $mg.L^{-1}$), thus long HRT results in long average time for (*RS*)-MCPP to remain in the system thus provide longer contact time between biomass and (*RS*)-MCPP. This would be likely to increase the specific (*RS*)-MCPP utilisation rates.

Through out Phase IV, the (*RS*)-MCPP concentration was maintained at 50 $mg.L^{-1}$ with concomitant decreases in the simpler carbon sources (from beer waste) as the OLR decreased, hence providing selective pressure on the microbial populations to utilise (*RS*)-MCPP as a carbon source. In this dual-substrate environment, catabolite repression of enzymes causes preferential degradation of the more easily metabolized substrates (in this case the simple molecules from beer wastewater) compared to the complex (*RS*)-MCPP. This phenomenon will be discussed in more detail Chapter 9.

Further speculation on the reason for gradual improvement in (*RS*)-MCPP degradation during the methanogenic phase (Phase IV), can be made on the basis of changing redox conditions. It is likely the redox conditions changed in the reactor with time as a result of the step increases in applied HRT which caused concomitant reduction OLR producing relatively less reducing conditions with time. These lower redox conditions may have provided an improved environment for (*RS*)-MCPP degradation and specific utilisation rate, however

further investigation is needed to confirm the possible link between redox conditions and (*RS*)-MCPP degradation rate.

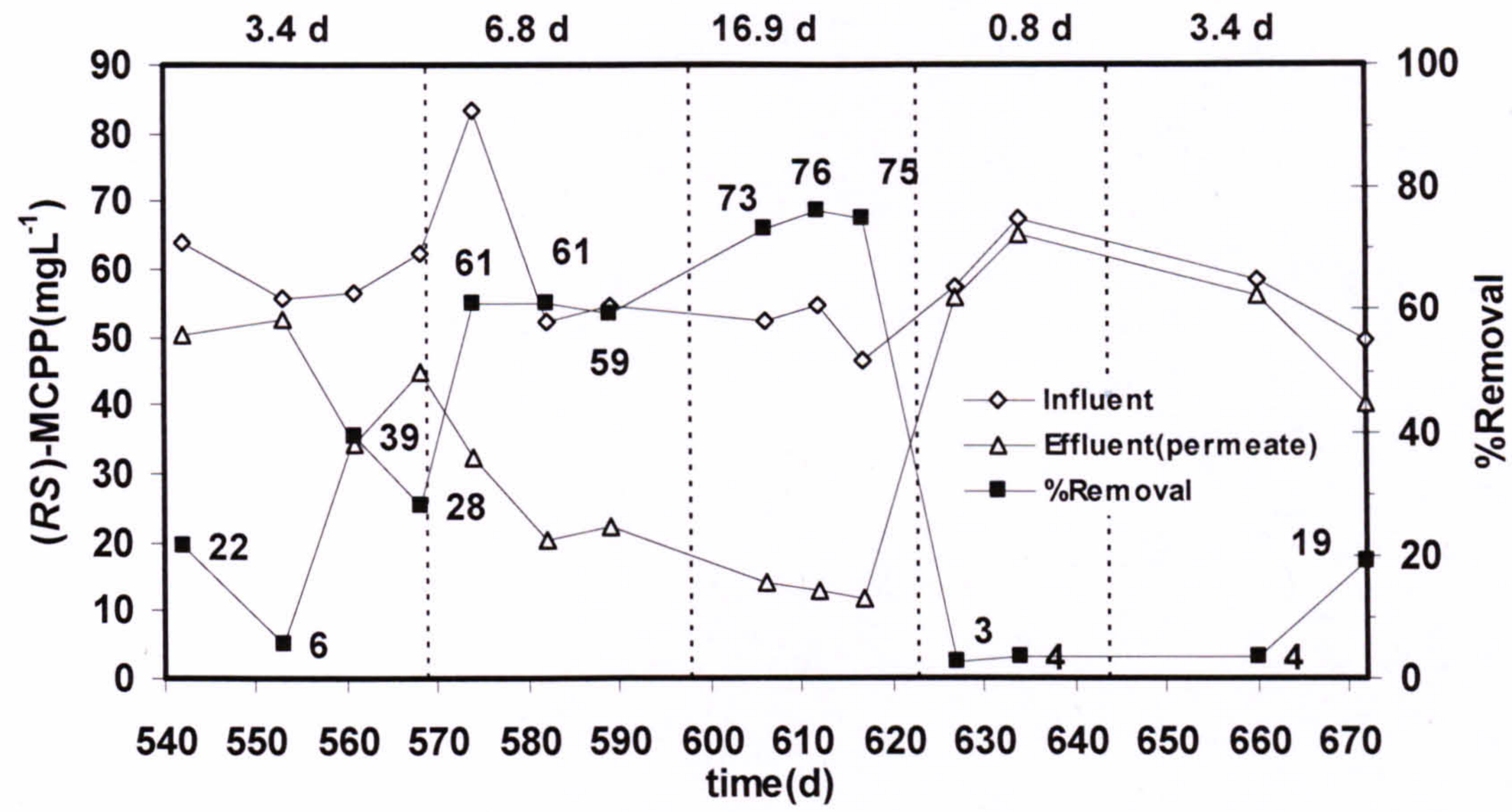


Figure 8.7: Changes in (*RS*)-MCPP removal efficiency in the AMBr at different HRT.

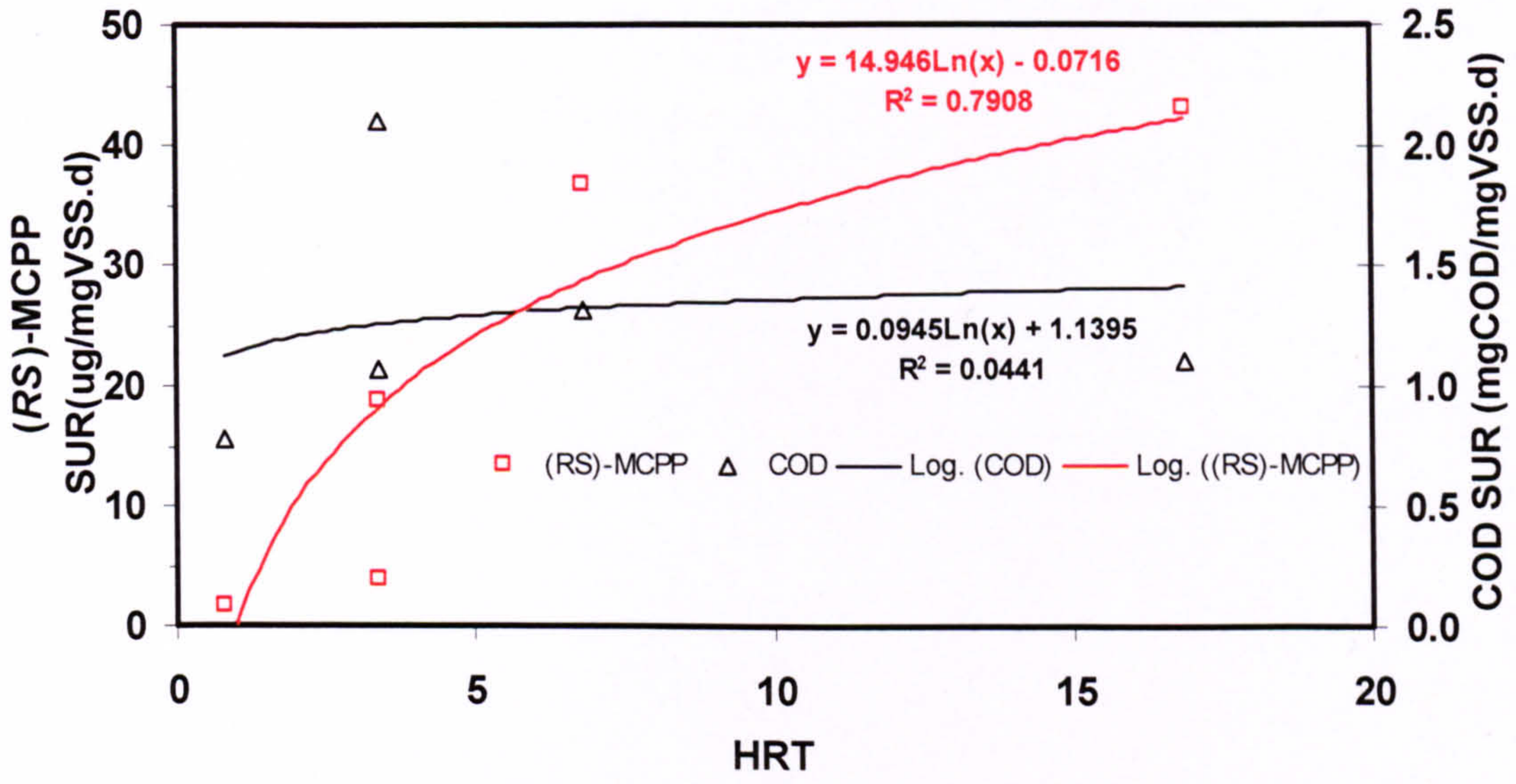


Figure 8.8: Specific COD and (*RS*)-MCPP utilisation rates in the AMBr at different HRT.

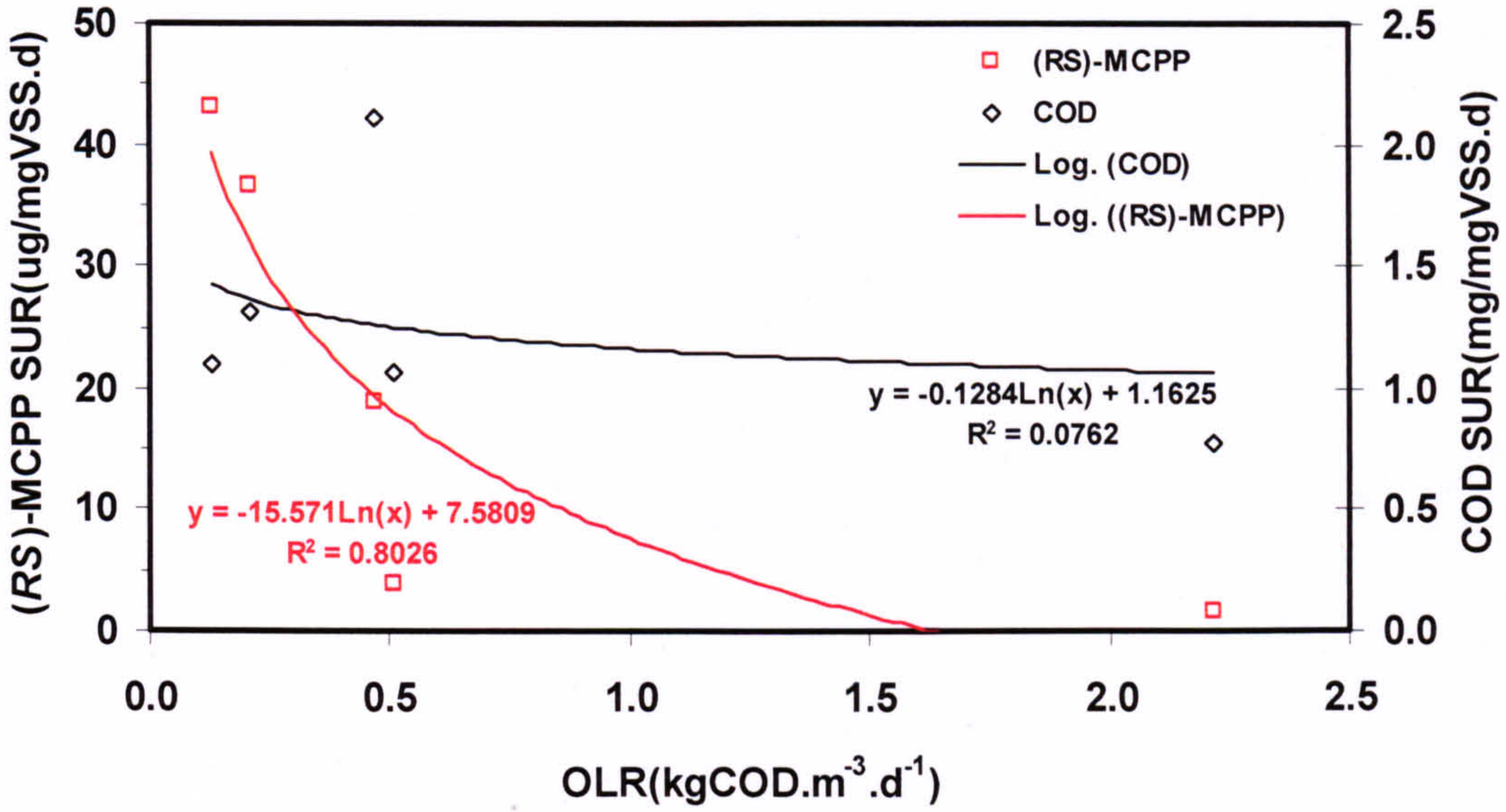


Figure 8.9: Specific COD and (*RS*)-MCPP utilisation rates in the AMBr at different OLR.

8.3.6 MLVSS & MLSS

Generally, biomass concentration peaks were clustered around a concentration less than 1500 mg.L^{-1} when operating at HRT of 3.4, 6.9 and 16.9 d, however, these increased rapidly to around 4000 mg.L^{-1} during hydraulic shock load (HRT 0.8 d). This was probably due to the higher OLR (2.22 kgCOD.m^{-3}.d^{-1}) operating during the hydraulic shock load compared to OLR less than 0.5 kgCOD.m^{-3}.d^{-1} operating before and after the hydraulic shock load (less than 0.5 kgCOD.m^{-3}.d^{-1}). High OLR provides high substrate to biomass resulting in increased gas production (Figure 8.6) and increased biomass growth. Low SS detected in the effluent (below 40 mg.L^{-1}) indicates the membrane formed an effective barrier allowing biomass and treated effluent is separated to a high degree of efficiency.

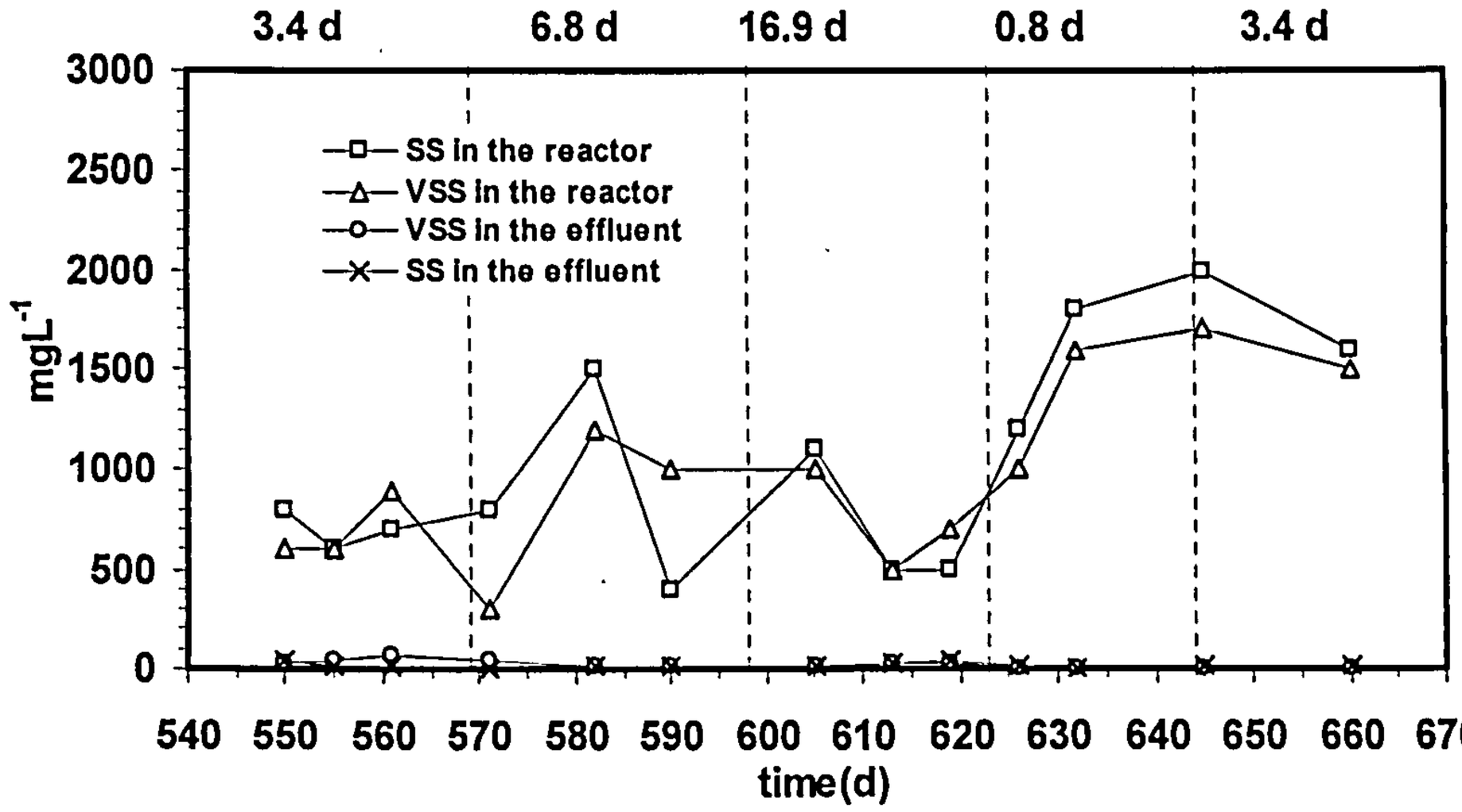


Figure 8.10: Biomass concentration (MLVSS) of the AMBr at different HRT.

8.4 RESULTS AND DISCUSSION (Phase V)

pH

The pH levels generally followed a similar pattern as in Phase IV when the pH level in the reactor and effluent gradually increased as HRT was increased from 3.4 d to 16.9 d (Figure 8.11). However, when the effluent tank was made strictly anaerobic by providing a nitrogen gas atmosphere in the effluent tank, the pH levels gradually dropped from pH 7.3 to pH 6.7 in 21 d. There was no substantial difference in total VFA (Figure 8.13) before and after introducing this nitrogen atmosphere, however CO_2 composition in biogas (Figure 8.15) showed a substantial increase from 2(±3) % to 7(±1) %. According to Speece, (1996), CO_2 levels as low as 1 to 3 % in the biogas (0.01 to 0.03 atmosphere of partial pressure) have a significant impact on the pH of water in equilibrium with it and

thus in this case, accounted for reduction of pH due to increased CO_2 composition in the biogas produced.

COD Removal

The removal efficiency was not considered to have been affected by the trace oxygen in the headspace of the effluent tank during Phase V because the COD removal efficiency remained at the same rate in Phase V after the N_2 atmosphere was introduced, 93.4(±1.2) % in Phase IV compared to 93.2(±3.01) % obtained in Phase V when nitrogen overpressure was maintained in the effluent tank for 19 d at HRT 16.9 d (Figure 8.12). The slight drop in COD efficiency on day 730 (Figure 8.12) was probably due to a slight drop in OLR which was not intentional but caused by error in feed make-up.

VFA

There was only transient effect observed when total VFA in the reactor gradually increased to 24.77 $mg.L^{-1}$ immediately after nitrogen was introduced in the effluent tank (Figure 8.13) and resulted in a substantial decrease in the reactor and effluent pH level (Figure 8.11). The reason for the transient increase is not clear, and could be due to population acclimatisation with cells adapting to a new pH.

Biogas

When a nitrogen atmosphere was introduced into the effluent reservoir system, methane composition transiently dropped from 45.3 % to 35.7 % but remained stable at 47 % after 11 d (Figure 8.15). In contrast, CO_2 composition increased

gradually from 2.9 % to 7.8 % within 21 d. The reason for this is because in Phase V, CO_2 was not lost into atmospheric air in the effluent reservoir (due to the N_2 overpressure) which resulted in CO_2 being contained (not lost) within the system.

(*RS*)-MCPP Degradation

The objective of conducting Phase V was to investigate the effect of trace oxygen in the headspace of the effluent tanks on (*RS*)-MCPP degradation since several studies have shown that (*RS*)-MCPP can degrade aerobically but not anaerobically (Lyngkilde and Christensen, 1992; Vink and van der Zee, 1997; Harrison et al., 1998; Rügge et al., 1999; Zipper et al., 1999). There was no substantial difference in (*RS*)-MCPP degradation in Phase V at HRT 16.9 d (with nitrogen atmosphere in the effluent tank) for 19 d of operation, compared to (*RS*)-MCPP degradation in Phase IV. The average (*RS*)-MCPP degradation rate in Phase V was 68 % (Figure 8.16) compared to 70 % in Phase IV (Figure 8.7), confirming that (*RS*)-MCPP degradation had not been elevated through aerobic metabolism as a result of trace levels of oxygen from the headspace of the effluent tank being carried to the reactor by permeate recycling.

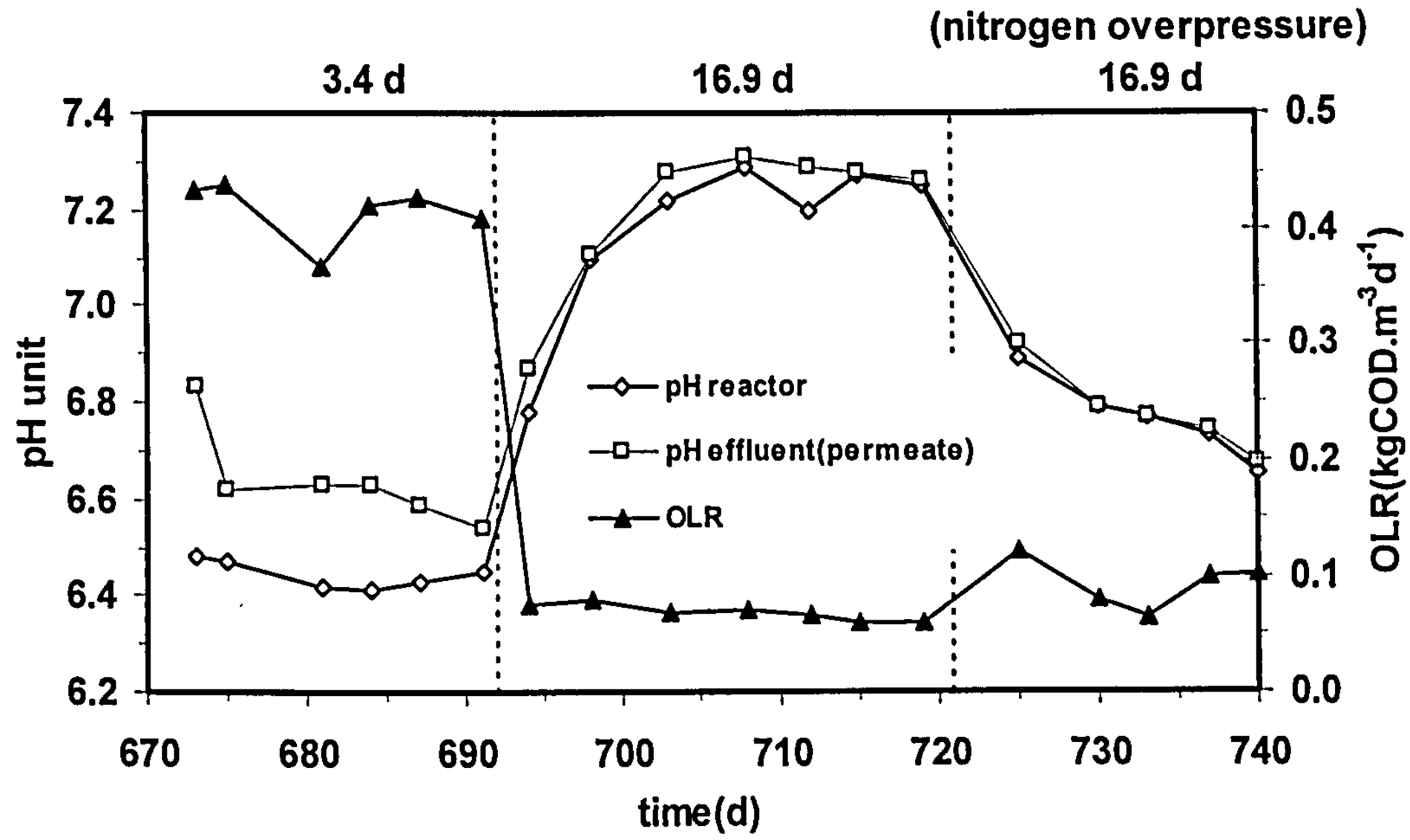


Figure 8.11: pH in the AMBr and effluent at different HRT in Phase V.

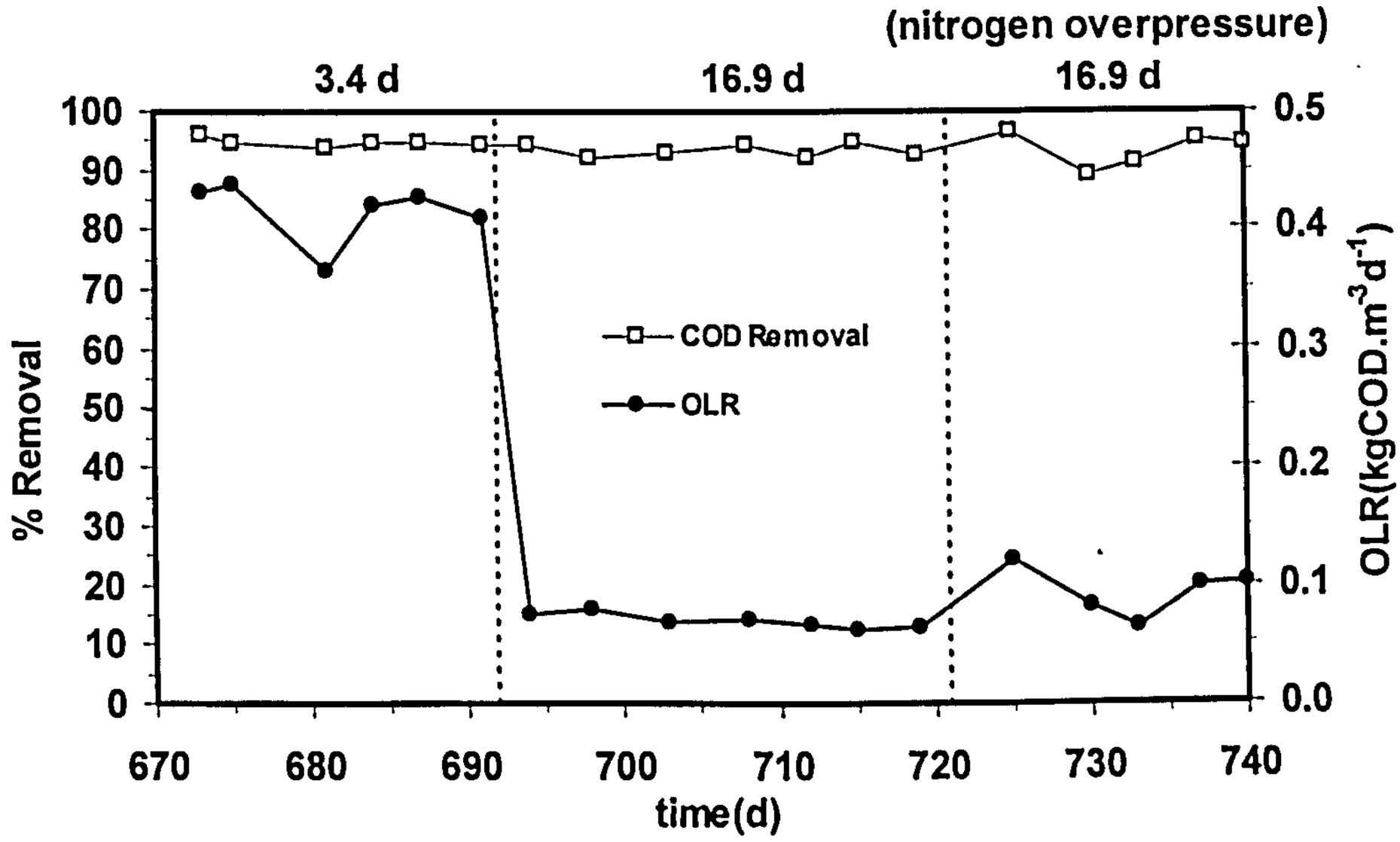


Figure 8.12: COD removal efficiency of the AMBr at different HRT in Phase V.

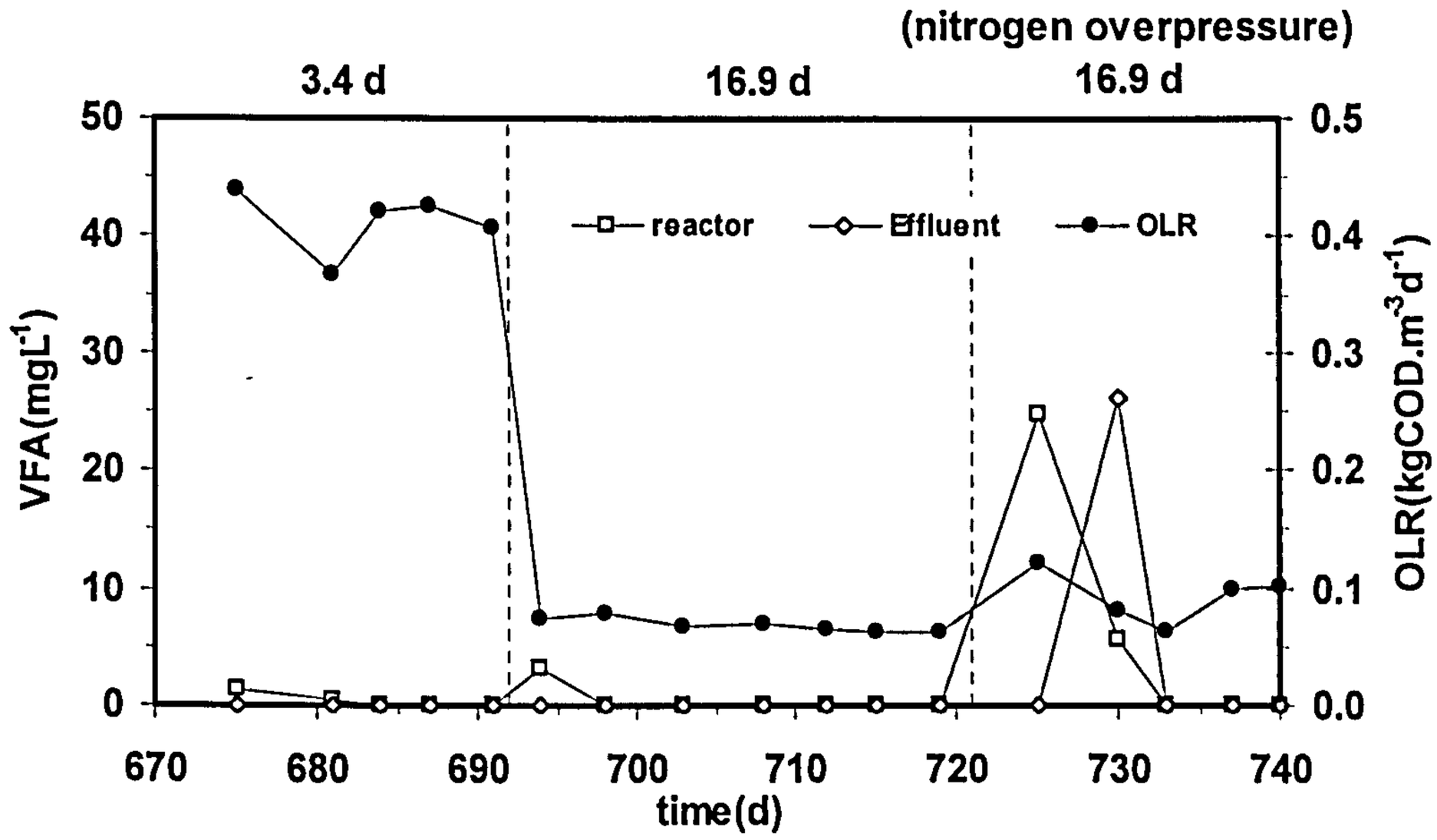


Figure 8.13: VFA concentration in the AMBr MLVSS and effluent at different HRT in Phase V.

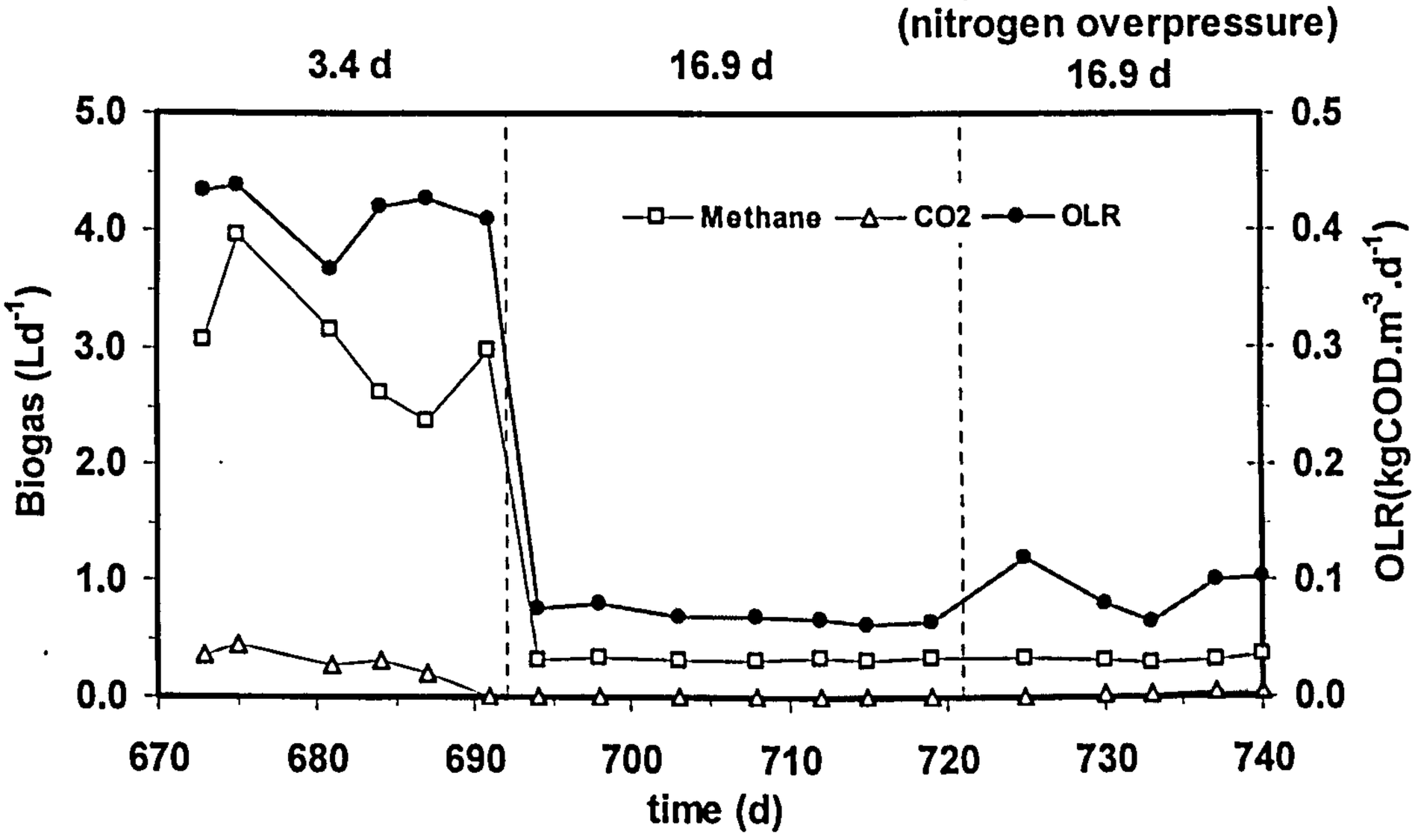


Figure 8.14: Biogas production of the AMBr at different HRT in Phase V.

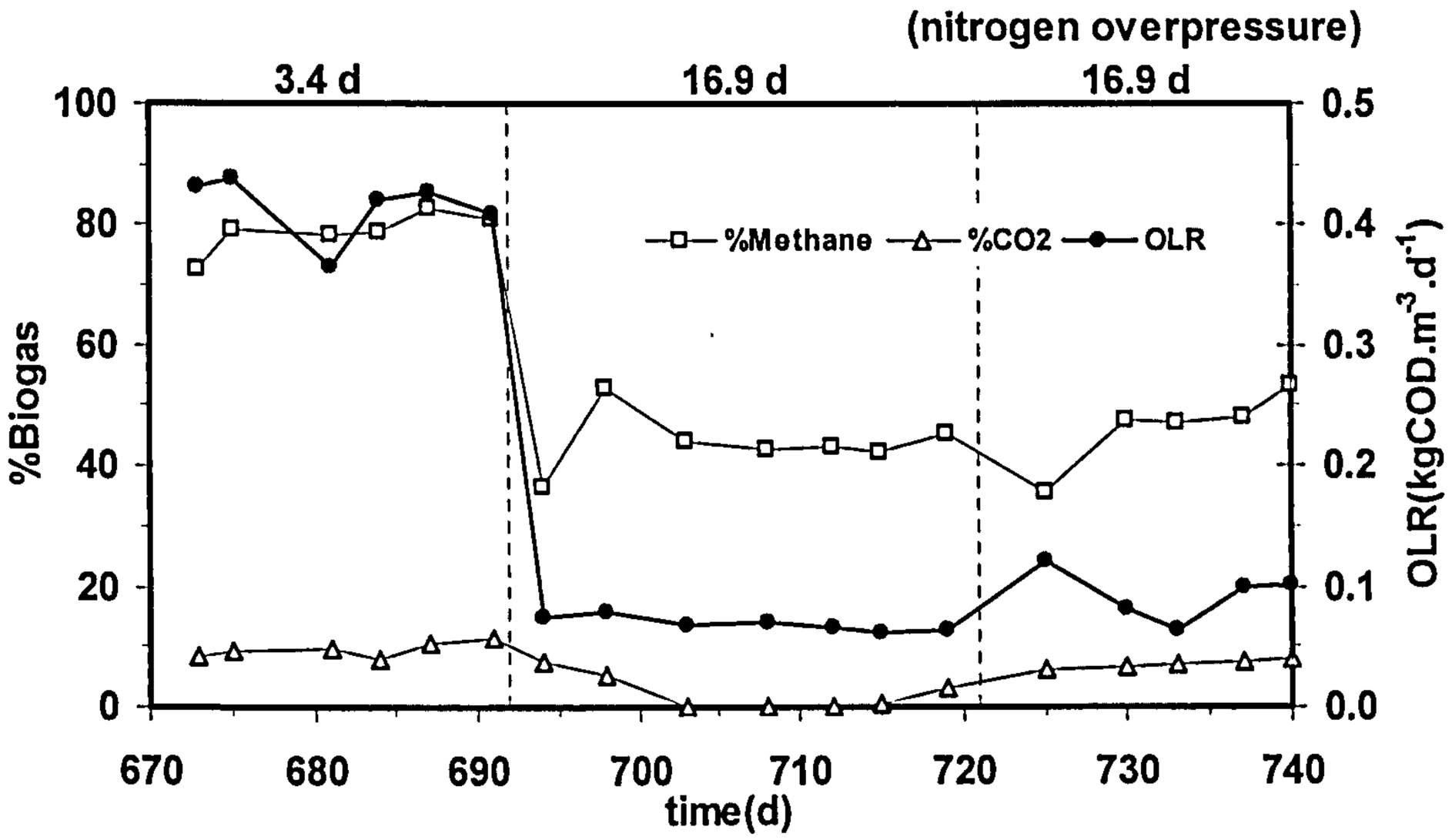


Figure 8.15: Biogas composition of the AMBr at different HRT in Phase V.

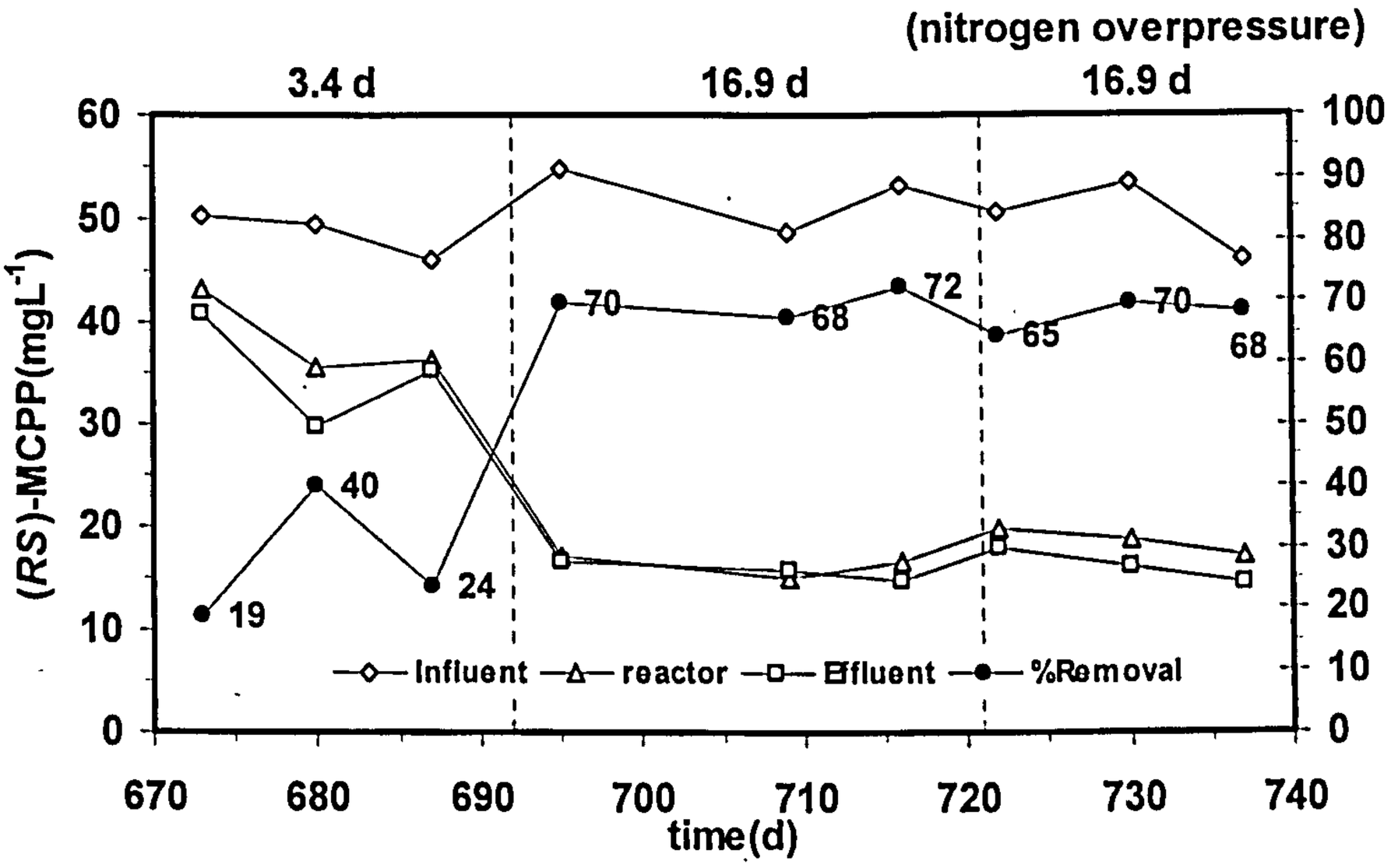


Figure 8.16: (*RS*)-MCPP concentration of the AMBr at different HRT in Phase V.

8.5 CONCLUSION

Based on the experimental results obtained in Phase IV, it can be concluded that:

- (*RS*)-MCPP SUR was dependent on HRT and/or OLR, the likely reasons being increased contact time between biomass and (*RS*)-MCPP (HRT effect), and/or gradual selective enrichment of microbial populations utilising (*RS*)-MCPP as the OLR decreased (OLR effect);

- the AMBr can contribute significantly to the removal of (*RS*)-MCPP in the synthetic wastewater with up to 70 % removal under high HRT;

- the AMBr was stable to a large transient hydraulic shock loads (twentyfold decrease in HRT) and it recovered rapidly to baseline performance after the hydraulic shock load had ended;

- low operating OLR or much diluted wastewater caused more than 50 % of the methane yield to leave the reactor by methane dissolving in the effluent (methane stripping);

- phase V showed that there was no effect of trace oxygen in the headspace of the effluent tank on system performance as biodegradation efficiency of (*RS*)-MCPP was the same for both Phase IV and V. This confirms that the (*RS*)-MCPP was efficiency degraded in the AMBr under stable methanogenic conditions; confirming O_2 is not an influence in the degradation of (*RS*)-MCPP in the AMBr system in Phase IV.

CHAPTER NINE

EFFECT OF DIFFERENT REDOX CONDITIONS ON (*RS*)-MCPP DEGRADATION AND THE BACTERIAL ECOLOGY OF THE AMBr

9.1 INTRODUCTION

Fluorescent *in situ* hybridisation (FISH) techniques using oligonucleotide probes to detect strain specific 16 rRNA has become common to quantify microbial composition of sludge (Amann et al., 1995). This quantitative technique was used in the current study to investigate, how different terminal electron acceptors affect the microbial populations present in the AMBr sludge.

At the end of this chapter, all relevant data on (*RS*)-MCPP utilisation rate in each phase are summarised and discussed in the context of the AMBr operating conditions.

9.2 METHODS

The protocols described in Section 4.6.11 were used for hybridization experiments. The samples were fixed in 4% paraformaldehyde immediately after sampling from the reactor, subsequently washed in phosphate buffer saline (PBS) and preserved at -20°C. These samples can be stored long-term between 6 months to a year until analysis is carried out. Total cell counts using 4', 6 diamidino-2-phenylindole (DAPI) were performed immediately after sampling according to the methods outlined in section 4.6.10. The population dynamics were evaluated by counting the total number of cells in the reactor at (*RS*)-MCPP

concentration of 5 mgL^{-1} and 200 mgL^{-1} on day 154 and 252 respectively (Phase I), COD/N-NO_3^{-1} ratio of 1 and 0.2 on day 360 and 415 respectively (Phase II), COD/SO_4^{-2} ratio of 2 and 0.2 on day 470 and 539 respectively (Phase III) and at HRT of 3.4, 6.8 and 16.9 d on day 569, 589 and 614 respectively (Table 9.1) after the system had reached steady state conditions. During microscopic analysis, means were calculated from 20 random chosen fields of view (FOV) for each sample. DAPI-staining was used to quantify the relative proportion of the bacterial and archael cells.

A range of 16S rRNA oligonucleotide probes were used in this investigation. These probes and their target groups are listed in Table 4.9. Procedures for FISH are given in Section 4.6.11. Cells were visualised using a Zeiss Standard Microscope 14 (Carl Zeiss) or confocal laser scanning microscope (CLSM) as described in section 4.6.15. The number of cells for each group specific probe was determined and means were calculated from 10 randomly chosen FOV for each sample. This data was used to calculate percentages of each specific group cells, relative to the total DAPI-stained cells. Statistical analysis for valid cell counting was determined according to Davenport and Curtis (2004) as described in Section 4.7. An example of the DAPI counting and percentage calculations for one specific group is given in Appendix II.

Table 9.1: Summary of reactor sampling schedule of AMBr system during molecular analysis.

Phase	Day	HRT(d)	(*RS*)-MCPP ($mg.L^{-1}$)	Redox Condition	COD ratio
I	154	3.3	5	Methanogenic	-
	252	3.3	200		-
II	360	3.3	50	Nitrate	1
	415	3.3	50	Reducing	0.2
III	470	3.3	50	Sulfate	2
	539	3.3	50	Reducing	0.2
IV	569	3.4	50	Methanogenic	-
	589	6.8	50		-
	614	16.9	50		-

9.3 RESULTS AND DISCUSSION

9.3.1 Microbial Community Structure in the AMBr Sludge

9.3.1.1 Total Cell Counts (TCC)

The cell counts using 4', 6 diamidino-2-phenylindole (DAPI) were visualised by epifluorescence microscopy (Figure 9.2) and the results are presented in Figure 9.1. In Phase I, there were generally no significant differences (i.e. $P > 0.05$) in the total number of cells observed when the concentration of (*RS*)-MCPP was increased from 5 $mg.L^{-1}$ to 200 $mg.L^{-1}$, even though it had been observed earlier in Section 5.3.3 that a (*RS*)-MCPP concentration of 200 $mg.L^{-1}$ caused an increase in effluent COD due to reduced performance of bacteria within the system. However, because one of the drawbacks in this staining technique is that it potentially fails to differentiate between living and dead cells (Madigan and Martinki, 2006) therefore the number of cells identified at (*RS*)-MCPP concentration of 200 $mg.L^{-1}$ would have contained a large number of inactive or dead cells.

However, when the redox environment was changed from methanogenic to nitrate reducing, the number of cells were significantly different (i.e. $P < 0.05$) changing from 6.46×10^9 per ml (Phase I at *(RS)*-MCPP=200 $mg.L^{-1}$) to 2.45×10^9 per ml (Phase II at COD/N-NO_3^{-1} ratio of 1). This reduction is most likely due to growth substrate deficiency after the OLR of the reactor had been reduced gradually from 1.52 $kgCOD.m^{-3}.d^{-1}$ (Phase I) to 0.45 $kgCOD.m^{-3}.d^{-1}$ (Phase II) affecting substrate availability and leading to a change in the microbial populations. A major reduction (10 fold) in the microbial population was observed when the COD/N-NO_3^{-1} ratio was further reduced to 0.2 resulting in a decline in the total number of cells to 2.45×10^8 per ml.

In contrast, there were generally no significant differences (i.e. $P > 0.05$) in the total number of cells when COD/SO_4^{-2} ratio was reduced from 0.4 to 0.2 in Phase III. In Phase IV, the total number of cells showed no significant differences (i.e. $P > 0.05$) when comparisons were made in the number of cells in each successive HRT.

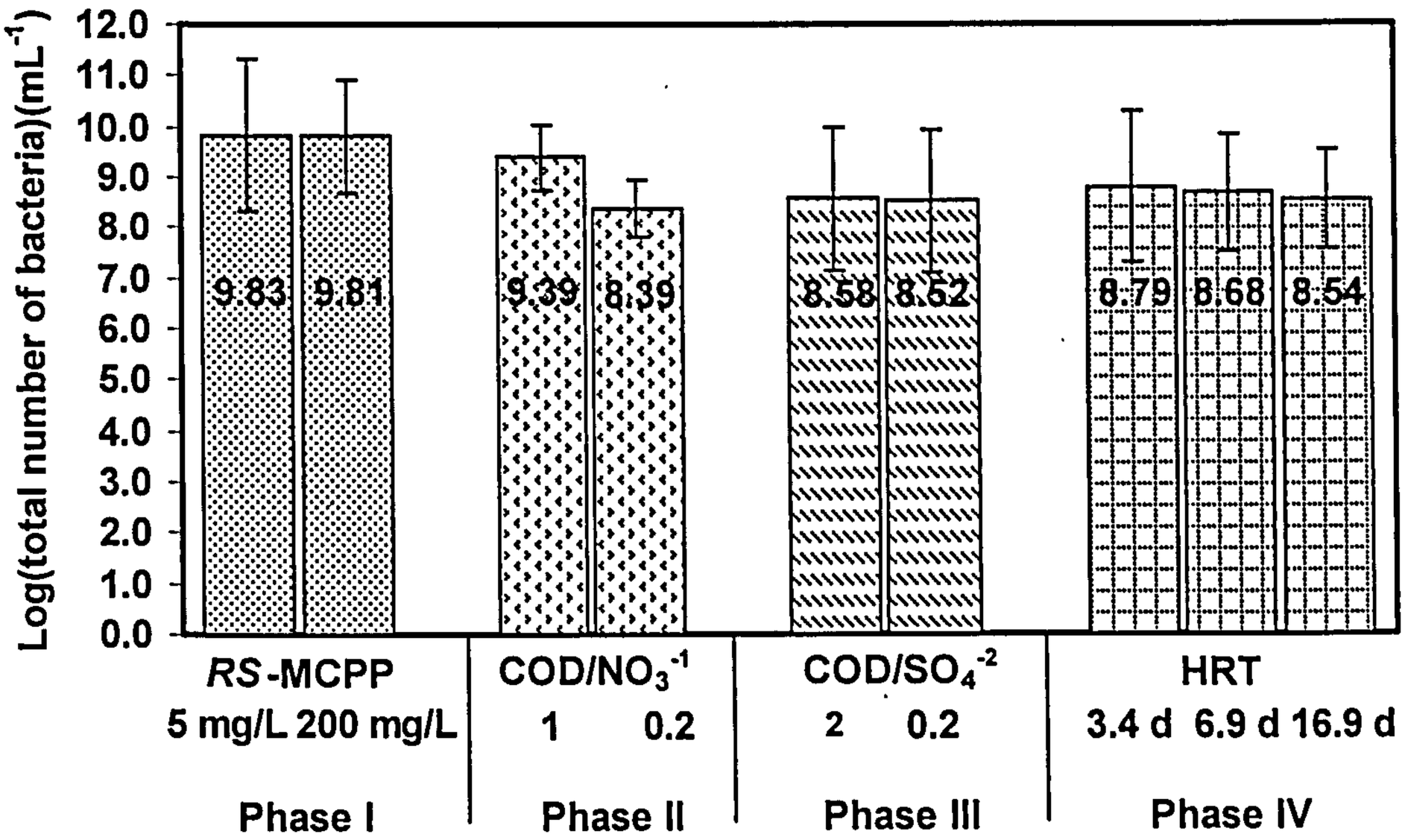


Figure 9.1: Mean total number of cells counted using DAPI in Phase I, II, III and IV. Means were calculated from 20 randomly chosen fields on one sample.

*numbers in bars are logarithm values (9.83 = 6.76×10^9 per ml; 9.81 = 6.46×10^9 per ml; 9.39 = 2.45×10^9 per ml; 8.39 = 2.45×10^8 per ml; 8.58 = 3.80×10^8 per ml; 8.52 = 3.31×10^8 per ml; 8.79 = 6.17×10^8 per ml; 4.79×10^8 per ml; 8.54 = 3.47×10^8 per ml).

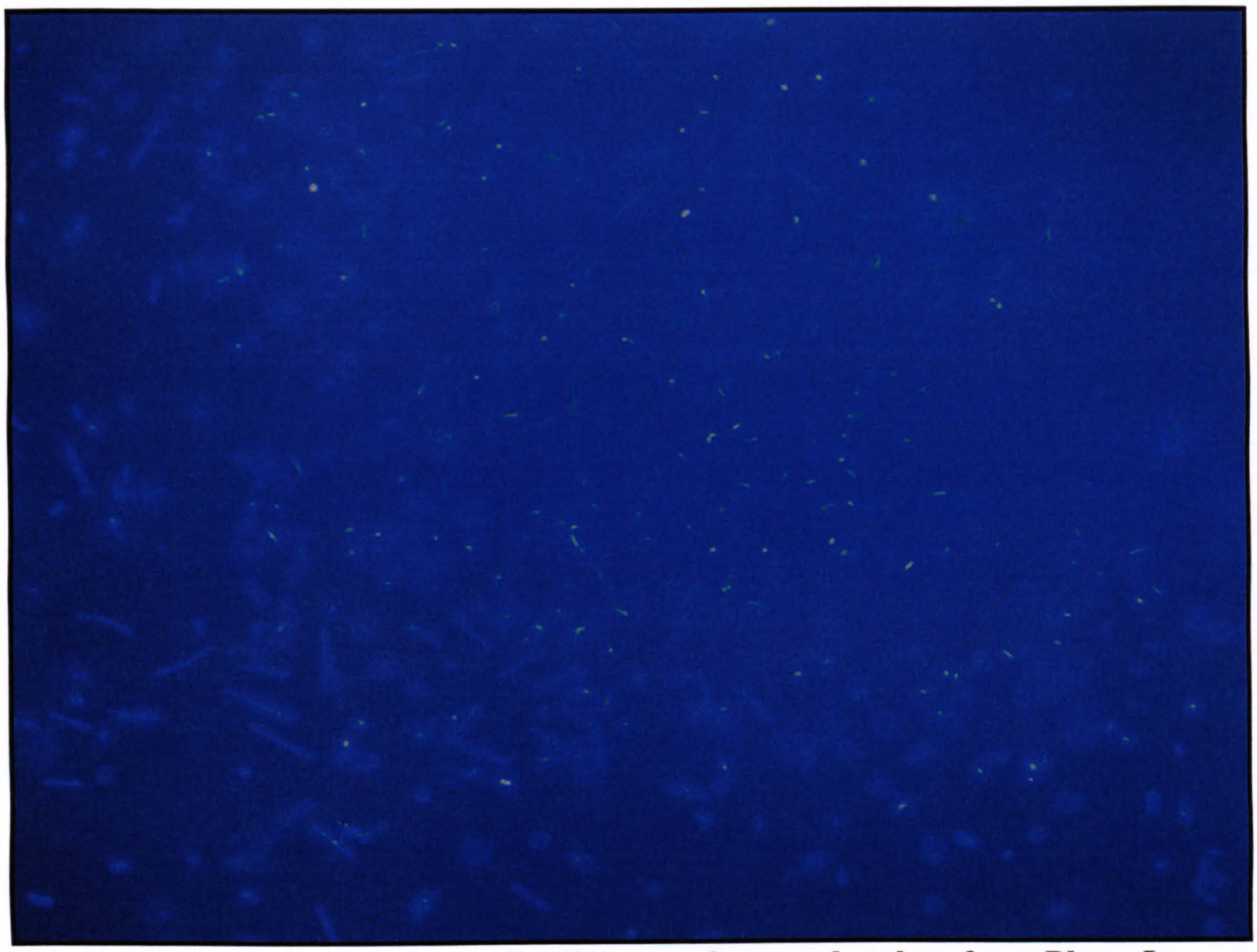


Figure 9.2: DAPI image for total cell count of a sample taken from Phase I on day 252 at (*RS*)-MCPP concentration of 200 mg.L^{-1}. Cells appear as fluorescent bright blue colour and means were calculated from 20 randomly fields of view (FOV) from one sample.

9.3.1.2 Comparison between Archael and Bacterial Populations

Hybridisation using universal bacterial probe EUB338 and archae probe ARC915 revealed the distribution of these phyla in each phase on a selected day (Table 9.1). The predominance of archael cells was obvious in all methanogenic phases (Phase I and IV) except at HRT 3.4 d on day 569 (early stage of Phase IV). However, within Phase II, the results start to show definite population shift, with a predominance of bacterial cells, 69.79(±25) % was observed at COD/N-NO_3^{-1} ratios of 0.2. This was due to higher bacterial yields using nitrate compared to CO_2 as electron terminal acceptor (0.534 gVSS.g^{-1} and 0.208 gVSS.g^{-1}, respectively) and a slower generation time (doubling time 16 times

faster for nitrate than for CO_2 -;Barber &Stuckey, 2000), and therefore resulted in definite population shift, which was also supported by increased in reactor COD (Section 6.3.2).

The number of cells detected with EUB338 probe continued to increased to 85.9(±11) % in Phase III at COD/SO_4^{-2} ratio of 2 before declining to 70.03(±25) % at a COD/SO_4^{-2} ratio of 0.2 due to insufficient COD being available (refer Section 7.3.2). Thus, archaea cell numbers decreased in the AMBr from 69.74(±25) % at COD/N-NO_3^{-1} ratios of 1 (Phase II) to 18.88(±8.5) % at COD/SO_4^{-2} ratio of 2 (Phase III).

Conversely, in Phase IV, the relative concentrations of archaea cell was 27.91(±17.1) %, 54.74(±23) % and 63.6(±35.2) % for HRT 3.4 d, 6.8 d and 16.9 d respectively, showing a steady increase, even though the OLR declined over this period, 0.47 kgCOD.m^{-3}.d^{-1}, 0.21 kgCOD.m^{-3}.d^{-1} and 0.13 kgCOD.m^{-3}.d^{-1} respectively. A similar finding was reported by Chelliapan (2006) when he found a reduction in the relative archaea population at reduced HRT (from HRT 4 d to 1 d) using a UASR treating Tylosin wastewater at a constant value of 200 mg.L^{-1} whilst maintaining a constant OLR.

The metabolic pathway of substrate degradation appears to influence the number of active cells present in a sludge sample (Díaz, et al., 2003). The relationship between bacteria and archaea depends on the type of substrate used (Díaz, et al., 2003). During Phase II and III, potassium nitrate and potassium sulphate were added to synthethic wastewater to encourage the growth of denitrifying and

sulphate reducing populations and this probably shifted the population to favour denitrifier and SRB growth. These results were supported by the pH data and the biogas production (Section 6.3.1 and 6.3.4 and Section 7.3.4) when the pH of the system reached a plateau at around pH 8.5 unit (optimum pH for denitrification-Phase II) at the same time that methane production dropped (Phase II and III).

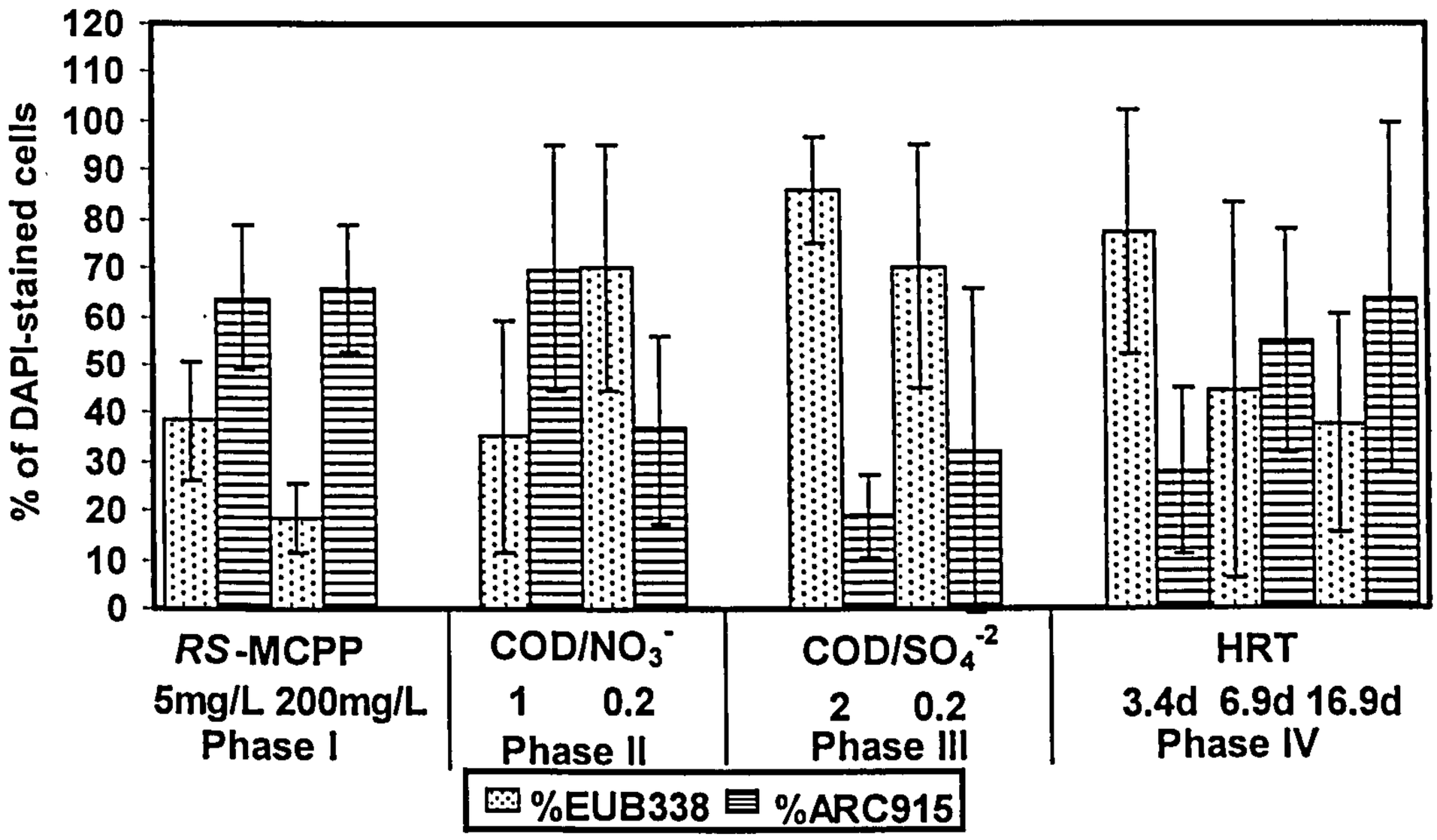
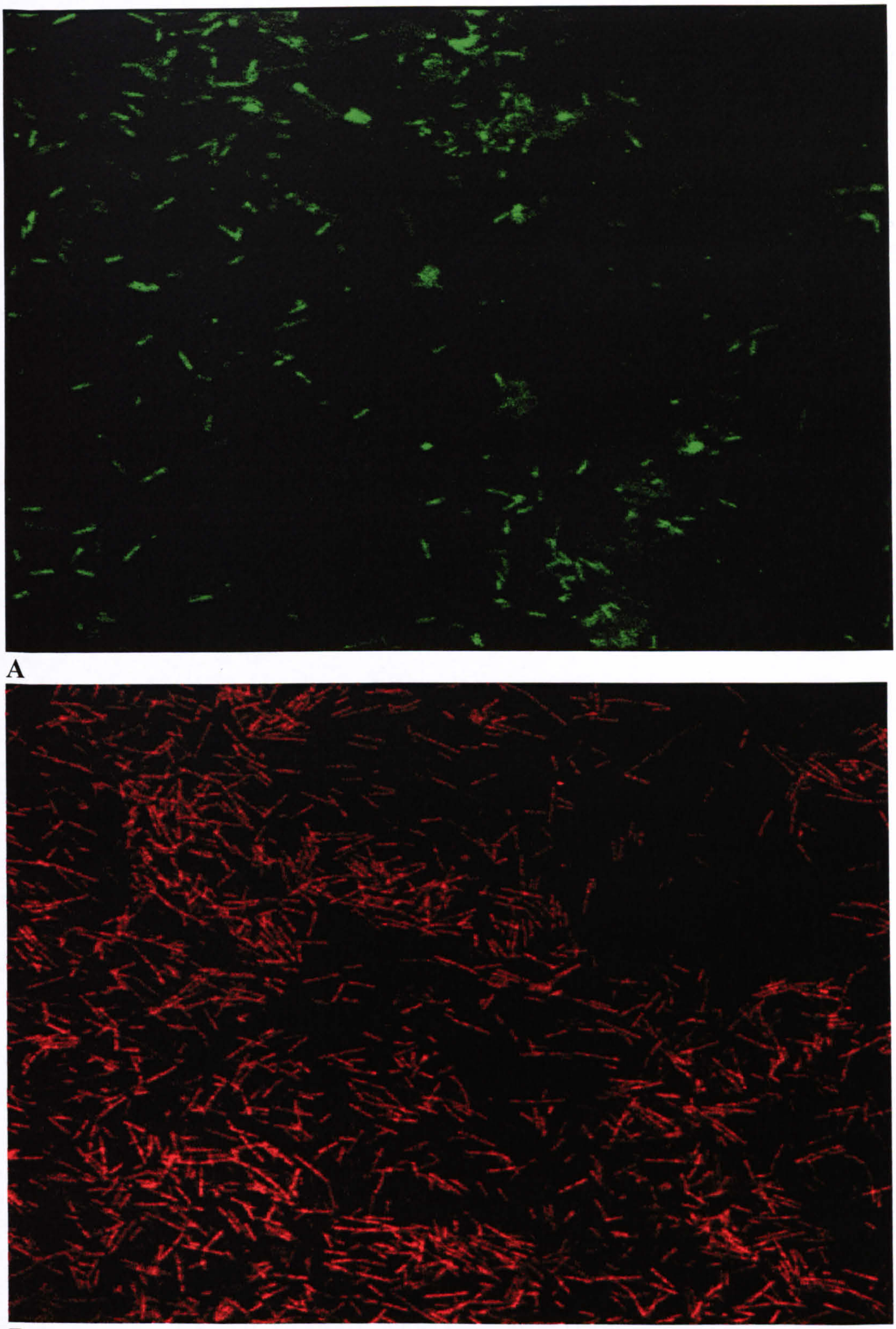


Figure 9.3: Percentages of DAPI-stained cells detected by FISH with probes for archaea (ARC915) and bacteria (EUB338) in each phase at selected (*RS*)-MCPP concentrations, COD/N-NO_3^{-1} ratios, COD/SO_4^{-2} ratios and HRT.



A

B

Figure 9.4: CLSM images of FISH samples taken from Phase II (nitrate reducing condition at COD/N-NO_3^{-1} ratio of 1) treating synthetic wastewater containing (*RS*)-MCPP at concentration of 50 mg.L^{-1} on day 360, showing the same field probe with A, EUB338 and B, ARC915.

9.3.1.3 Composition of the Archael Population

The characteristic morphology of the acetoclastic methanogens, *Methanosaeta* and *Methanosarcina* were not clearly visualized using the archael probe (ARC915). By using more specific probes such as MX825 and MS821, these cells were hybridized and were used to gain insight into methanogens making up the archael population in the AMBr system. It is well documented elsewhere (Rocheleau et al., 1999; Speece, 1996) that acetoclastic methanogens which utilise acetate play an important role in anaerobic processes. *Methanosaeta* has a high affinity for acetate, K_s = 20 $mg.L^{-1}$, but relatively low maximum specific utilization rate, k_{max} = 2 to 4 $gCOD.gVSS^{-1}.d^{-1}$ (Speece, 1996). On the other hand *Methanosarcina* has a much lower affinity, K_s = 400 $mg.L^{-1}$, but a higher maximum specific utilization rate, k_{max} = 6 to 10 $gCOD.gVSS^{-1}d^{-1}$ (Speece, 1996). These parameters affect population numbers in relation to available substrate concentration.

The changes in population were determined in each phase on selected days (Table 9.1). The mean percentages of these two specific archaea were calculated from 10 randomly chosen fields on each well of the sample spot on the slide and the sum of the counts obtained from each specific probe was always less than 100% of the total ARC915 count, indicating the presence of archael cells not detected using these two specific probes. Figure 9.5 shows that *Methanosaeta* cells hybridised with MX825 were the major acetoclastic methanogens present in all Phases (I, II, III and IV) of the reactor. This is supported by the low concentration of acetic detected (Section 5.3.4, 6.3.3, 7.3.3, and 8.3.3) resulting from high COD removal efficiency within the reactor throughout the experiment

promoting the presence of *Methanosaeta*-related species over *Methanosarcina* as the main acetate utilizing methanogens.

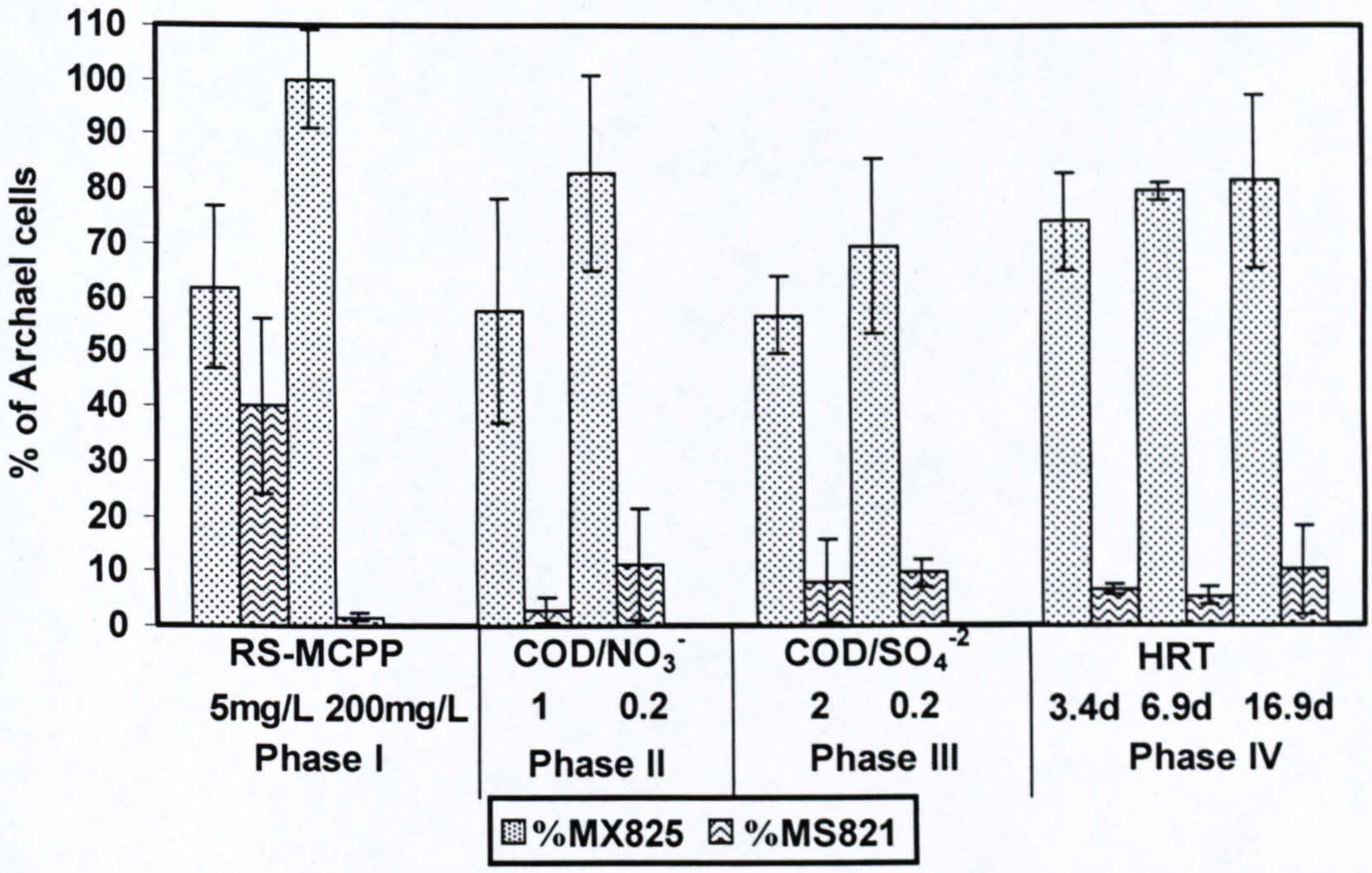


Figure 9.5: Archael community analysis sampled at selected (*RS*)-MCPP concentrations, COD/N-NO_3^{-1} ratios, COD/SO_4^{-2} ratios and HRT, showing count obtained using probes MX825 (*Methanosaeta*) and MS821 (*Methanosarcina*) expressed as percentage of total archael population (probe ARC915).

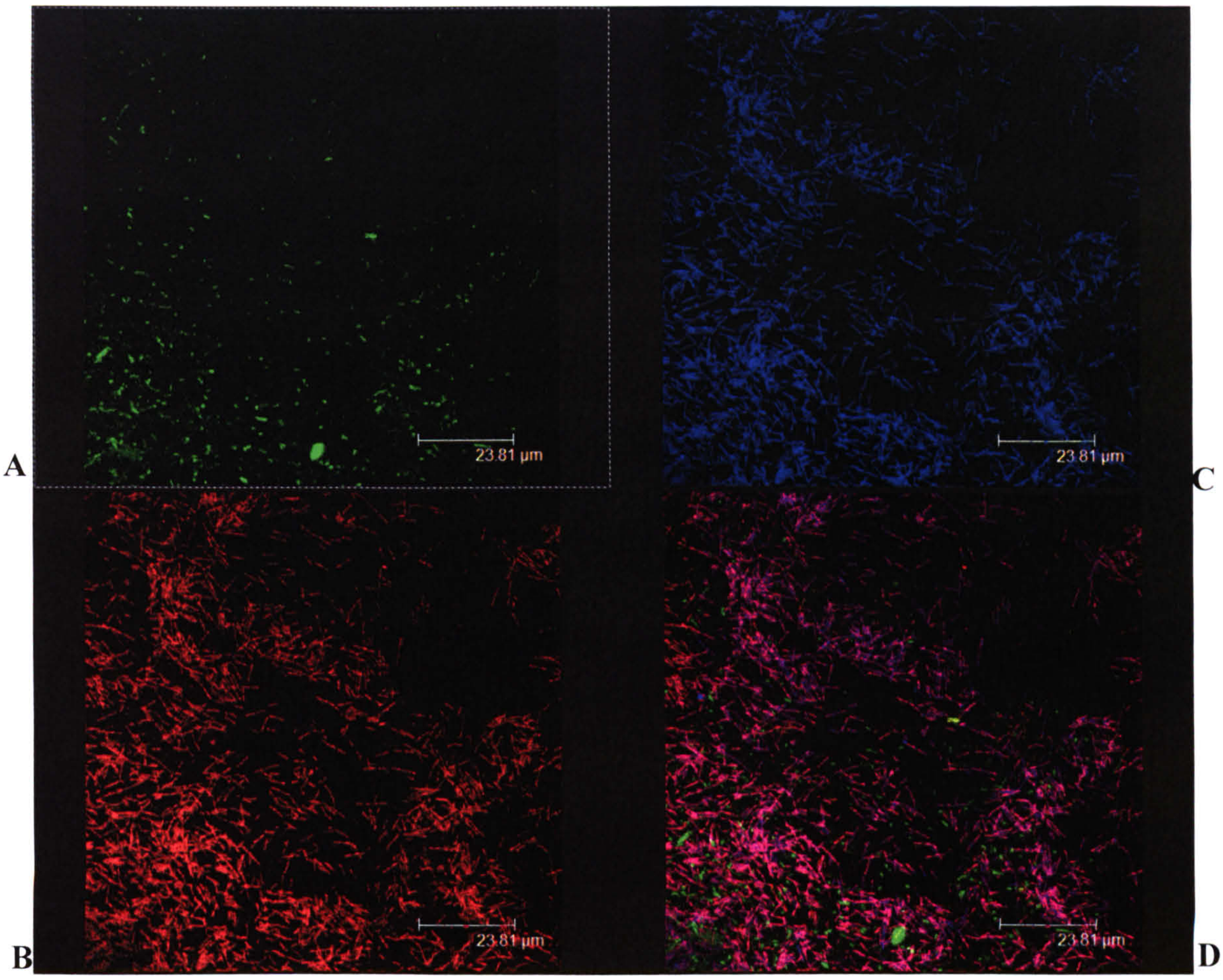


Figure 9.6: CLSM images from FISH analysis of a sample taken from Phase II (nitrate reducing at COD/N-NO_3^{-1} ratio of 1) treating synthetic wastewater containing (*RS*)-MCPP at concentration of 50 mg.L^{-1} on day 360, showing the same field probed with A, EUB338; B, ARC915; and C, MX825. A Cy3-labeled probe (emits as red) specific for archaea (ARC915) is used in combination with a more specific probe (MX825) labelled with Cy5 (emits as blue). Only cells fluorescing magenta (D) would be identified as MX825.

9.4 COMPARISON ON SPECIFIC (*RS*)-MCPP UTILISATION RATES UNDER DIFFERENT REDOX CONDITIONS

Table 9.2: Comparisons on specific (*RS*)-MCPP utilisation rates under different redox conditions.

	Methanogenesis	Nitrate Reducing	Sulphate Reducing
INHIBITION	inhibition shown at (*RS*)-MCPP concentrations of 200 $mg.L^{-1}$	no inhibition shown	no inhibition shown
RELATIONSHIP BETWEEN (*RS*)-MCPP SUR & TEST PARAMETERS	(*RS*)-MCPP SUR proportional increases with (*RS*)-MCPP concentrations and HRT (R^2 = 0.98 and 0.80, respectively)	(*RS*)-MCPP SUR inversely proportional with COD/Nitrate ratios (R^2 = 0.67)	(*RS*)-MCPP SUR inversely proportional with COD/Sulphate ratios (R^2 = 0.67)
RELATIONSHIP BETWEEN (*RS*)-MCPP SUR & OLR	(*RS*)-MCPP SUR inversely proportional with OLR (R^2 = 0.80)	(*RS*)-MCPP SUR inversely proportional with OLR (R^2 = 0.96)	OLR fixed at 0.07 $kgCOD.m^{3-}d^{-}$
HIGHEST (*RS*)-MCPP SUR VALUE	43.2 $\mu g.mgVSS^{-1}.d^{-}$ at the highest HRT (16.9 days) & at the lowest OLR (0.13 $kgCOD.m^{3-}d^{-}$)	60.7 $\mu g.mgVSS^{-1}.d^{-1}$ at the lowest COD/Nitrate ratio (0.2) & OLR (0.07 $kgCOD.m^{3-}d^{-1}$)	29.6 $\mu g.mgVSS^{-1}.d^{-1}$ at the lowest COD/Sulphate ratio (0.2)
PREDOMINANCE CELLS	archae	shift from archae to eubacteria (COD/Nitrate 1 to 0.2)	eubacteria

9.4.1 Specific (*RS*)-MCPP Utilisation Rate (SUR)

A comparison of (*RS*)-MCPP utilisation rates in each phase of the research revealed wide ranges (*RS*)-MCPP SUR (Table 9.2), however, although changes were observed in the composition of the bacterial population (Section 9.3.1.2 and 9.3.1.3), no direct linkage could be made with any of the individual groups identified by FISH. Under methanogenic conditions, (*RS*)-MCPP SUR was proportionally increased with increases in (*RS*)-MCPP concentrations and HRT. An increase in (*RS*)-MCPP SUR with increasing (*RS*)-MCPP concentrations might be caused by acclimatisation and diversification of microbial populations leading to the selection of strains able to utilise (*RS*)-MCPP more effectively.

The long acclimatisation time required in this study was not unexpected as other studies on (*RS*)-MCPP have shown acclimation periods (lag phases) up to 35 days, under aerobic conditions (Torang et al., 2003; González et al., 2006). Moreover, anaerobic systems usually require longer acclimatisation times than aerobic ones (Speece, 1996).

There are two possible explanations for a progressive increase in (*RS*)-MCPP SUR with HRT; longer contact time between (*RS*)-MCPP and biomass; and sequential utilisation in dual-substrate conditions (simple COD needs to be degraded before (*RS*)-MCPP is utilised (see Section 8.3.5).

The sequential utilisation pattern observed here was due to the presence of two groups of substrate (simpler carbon and more complex carbon-(*RS*)-MCPP) which resulted in simpler carbon substrates being preferentially utilised. The

dual-substrate environment observed is in agreement with other observations reported in the literature when dual-substrates with different energy yields are metabolised in order of decreasing energy yield. Chin et al., 2005 and Papanastasiou & Maier, 1982 observed that when glucose and other phenoxy acids such as 2,4-D were present in a dual-substrate environment, glucose is utilised preferentially. This phenomenon, known as diauxic growth, is due to catabolite repression, which denotes the repression of enzymes that degrade a less rapidly metabolized energy source in the presence of a more rapidly metabolized one (Chang & Alvez-Cohen, 1995; Chin et al., 2005).

The relationship between (*RS*)-MCPP SUR and COD/nitrate and sulphate ratios were similar, SUR were inversely proportional to the ratios (at $R^2 = 0.67$). Higher (*RS*)-MCPP SUR observed in Phase II compared to Phase III suggests that nitrate reducing conditions were more efficient than sulphate reducing conditions in degrading (*RS*)-MCPP when operating at otherwise the same conditions (HRT and OLR). For instance, the highest SUR achieved at COD/nitrate ratio of 0.2 was 60.7 $\mu g.mgVSS^{-1}.d^{-1}$ compared to only 29.6 $\mu g.mgVSS^{-1}.d^{-1}$ (i.e. half the value in Phase II) at COD/sulphate ratio of 0.2.

This might be due to the greater ability of facultative denitrifiers, compared to sulphate reducing bacteria, to utilise (*RS*)-MCPP. Another possibility might be that this was due to different redox environments in the two phases which influenced the (*RS*)-MCPP SUR.

Furthermore, under anoxic conditions, the (*RS*)-MCPP SUR was inversely proportional to OLR (Phase II) (Figure 6.9), and as the COD from simpler organic carbon was limited (Phase III) the specific (*RS*)-MCPP utilisation was increased.

9.4.2 Inhibition of COD degradation

COD removal efficiency was inhibited under anaerobic conditions at (*RS*)-MCPP concentrations of 200 $mg.L^{-1}$ when its values dropped to 94 % compared to average values of 98 % at lower doses. In contrast, there was no apparent decrease in methane production (Section 5.5), and VFA which was mainly present as acetate was low (below 40 $mg.L^{-1}$) at this time. This suggests that methanogens were not inhibited by (*RS*)-MCPP and this hypothesis is reinforced by an observed increase in *Methanosaeta* cells that hybridised with MX825 from 62 % to 100 % (as percentage of total archael population). In addition to methanogens, there are other groups of bacteria which work together to degrade complex organic compounds in an anaerobic digester, hence bacteria other than methanogens might have been affected by (*RS*)-MCPP at this concentration but not detected by the FISH analysis.

In contrast, there was no inhibition of COD observed in Phase II at (*RS*)-MCPP concentrations of 200 $mg.L^{-1}$ when soluble COD removal efficiency at COD/N-NO_3^{-1} ratios of 250 and 8 was relatively constant and had an average removal over both ratios of 93(±0.6) %, however, the soluble COD removal efficiency showed a slight reduction to 90(±1.25) % and 83(±3) % at COD/N-NO_3^{-1} ratios of 3 and 1, respectively. This was probably due to the fact that the C/N ratio in

the influent was not optimal as OLR was decreased. The same observation was reported by Chiu and Chung (2003) when they observed a COD increase in the treated COD effluent when denitrification operated at a sub-optimal C/N ratio. This hypothesis is reinforced by high removal in nitrate (99 % removal) for COD/N-NO_3^{-1} ratios of 250 down to 1 observed in the current study.

Equally, no inhibition was observed in Phase III by (*RS*)-MCPP when COD being removed by the sulphate reduction, increased from 1 to 25 % at COD:SO_4^{-2} ratios of 2, and the COD efficiency further increased to an average of 75 % at ratios 0.4 and 0.2. Although the total COD removal efficiency was gradually dropped from 92 to 75 % throughout this phase, this was caused by the detrimental effect of sulphate reduction on methanogenesis as discussed in Section 7.3.2.

9.5 CONCLUSIONS

This study concluded that the use of 16S rRNA-targeted probes can be a very useful tool to study the microbial ecology in the AMBr anaerobic treatment system. Using this technique the following conclusions can be drawn.

- Using domain and group-specific FISH probes, significant differences were observed in the microbial populations that developed when the redox environments were shifted from methanogenic conditions to nitrate reducing conditions, (P-value = 0.000; < 0.05); from nitrate reducing to sulphate reducing conditions, (P-value = 0.000; < 0.05), however, no significant difference was observed in the population when changing from sulphate reducing to methanogenic conditions, (P-value = 0.096; < 0.05).

Furthermore, no significant differences were seen when the system was operated at a range of (*RS*)-MCPP concentrations (P-value = 0.723; > 0.05) and HRT (P-value = 0.104; > 0.05) under continuous methanogenic conditions;

- Composition of archaea and bacterial group distribution were influenced by redox environment. During methanogenic conditions, methanogenic archael populations dominated the microbial community in the reactor sludge, however, as the redox environment changed to denitrifying then sulphate reducing conditions, microbial community shifted to predominance of bacterial cells;
- *Methanosaeta* was identified as the predominant methanogenic archaea species irrespective of redox environment, (*RS*)-MCPP concentration or HRT;
- *Methanosaeta* was not adversely affected by (*RS*)-MCPP and grow well with all doses under tests;
- *Methanosarcina* grow in presence of all (*RS*)-MCPP concentration but was probably limited by growth kinetics rather than (*RS*)-MCPP concentration;
- A comparison of (*RS*)-MCPP utilisation rates in each phase of the research revealed wide ranges (*RS*)-MCPP degradation efficiency and (*RS*)-MCPP SUR, however, although changes were observed in the composition of the bacterial population, no direct linkage could be made with any of the individual groups identified by FISH;
- The long acclimatisation and sequential substrate utilisation pattern observed is in agreement with observations by others reported in the literature;

- When simple carbon substrates were limited, (*RS*)-MCPP was used by the acclimated biomass as a carbon source under all redox conditions;
- Inhibition was observed in the methanogenic phase at (*RS*)-MCPP concentrations of 200 $mg.L^{-1}$ but no inhibition was observed under nitrate reducing (Phase II) and sulphate reducing conditions (Phase III) at concentrations 200 and 50 $mg.L^{-1}$ respectively.

CHAPTER TEN

CONCLUSIONS AND RECOMMENDATIONS

10.1 DEVELOPMENT OF HPLC METHOD FOR THE ANALYSIS OF (*RS*)-MCPP

An HPLC method for (*RS*)-MCPP analysis in aqueous samples was successfully developed in the current study. An extensive series of development method experiments, such as selection of analytical column, selection of mobile phase and its working composition, pH and selection of optimum wavelength were carried out before the optimised HPLC conditions were identified.

10.2 CONCLUSIONS FROM EACH EXPERIMENTAL CHAPTER

10.2.1 Effect of elevated (*RS*)-MCPP concentrations on AMBr performance under methanogenic conditions – Chapter 5

Despite the observation that (*RS*)-MCPP was degraded in the AMBr soon after start-up (Chapter 5), other research has found a long adaptation period (lag-phase) is required before biodegradation occurs (see Section 5.3.6), a factor which would cause shock loads in real wastewater treatment plants to result in inefficient removal.

However, in the current research, increasing concentrations of (*RS*)-MCPP in the AMBr promoted a progressive increases in the (*RS*)-MCPP SUR, probably as a result of a gradual acclimatisation and diversification of bacterial populations more able to utilise (*RS*)-MCPP effectively.

The COD removal efficiency decreased gradually from 98 % to 94 % as the (*RS*)-MCPP concentrations increased in the feed from 50 – 200 $mg.L^{-1}$, respectively. This shows that (*RS*)-MCPP had a small but measurable inhibitory effect on COD removal. However, the methanogens were not inhibited by (*RS*)-MCPP, even at 200 $mg.L^{-1}$, because methane yields near to the theoretical values were observed at all (*RS*)-MCPP concentrations.

10.2.2 Effect of anoxic conditions (nitrate-reducing) on (*RS*)-MCPP degradation - Chapter 6

The AMBr has great potential for wastewater treatment in the future. The current research showed that optimum anaerobic treatment of wastewater containing (*RS*)-MCPP was achieved with acclimatised biomass under nitrate reducing conditions established with progressive reduction in the COD/N-NO_3^{-1} ratio and OLR.

Consequently, (*RS*)-MCPP removal efficiency and (*RS*)-MCPP SUR improved from 2 – 47 % and 0.5 to 60.7 $\mu g.mgVSS^{-1}.d^{-1}$, respectively, as the COD/N - NO_3^{-1} ratios and OLR were reduced from 250 to 0.2 and 1.51 to 0.07 $kgCOD.m^{3-}$ d^{-1}, respectively. Decreases in COD/N-NO_3^{-1} ratios could possibly have increased the facultative denitrifier population which overall improved the (*RS*)-MCPP SUR and (*RS*)-MCPP degradation efficiency. Another possible explanation could be the phenomenon of diauxic growth; catabolite repression which denotes the repression of enzymes that degrade a less readily metabolized energy source (i.e. (*RS*)-MCPP) in the presence of an easily metabolized substrate (i.e. COD from beer wastewater).

In addition, long acclimatisation could be another factor that contributes to gradual improvement in the (*RS*)-MCPP SUR and (*RS*)-MCPP degradation efficiency since other researchers have found that a long lag phase (up to 35 days) may be required before efficient degradation occurred under aerobic conditions.

This experiment also showed that methanogenesis and denitrification can be achieved simultaneously in a single reactor unit with three distinct types of substrate metabolism occurring under different conditions; predominantly methanogenic (COD : $N\text{-}NO_3^{-1}$ ratio ≥ 250), methanogenic and denitrification (3 ≤ $COD/N\text{-}NO_3^{-1}$ ratio ≤ 8) and exclusively by denitrification (COD : $N\text{-}NO_3^{-1}$ ratio ≤ 1). Under this range of COD : $N\text{-}NO_3^{-1}$ ratios, the greatest level of (*RS*)-MCPP degradation occurred at a ratio of 0.2.

To date, other published experiments conducted on (*RS*)-MCPP in microscom's have only brought about a basic understanding of (*RS*)-MCPP utilisation and degradation in the environment. By using AMBr, a clearer understanding of factors affecting the (*RS*)-MCPP specific utilisation rate, such as OLR, $COD/N\text{-}NO_3^{-1}$ ratios and acclimatisation have been shown. However, more research is required to investigate which factors are the most significant in determining (*RS*)-MCPP degradation and utilisation rate.

10.2.3 Effect of anoxic conditions (sulphate-reducing) (RS)-MCPP degradation - Chapter 7

(*RS*)-MCPP removal efficiency and SUR improved from 11 to 27 % and 8.3 to 36.0 $\mu g.mgVSS^{-1}.d^{-1}$, respectively, with decreases in COD/SO_4^{-2} ratios (2 to 0.4) over time. As the COD/SO_4^{-2} ratio decreased, there was a greater excess of sulphate remaining, above that required for simple carbon (beer wastewater) oxidation, so that sufficient sulphate remained to support (*RS*)-MCPP degradation by processes linked to sulphate reduction, and this resulted in higher (*RS*)-MCPP removal efficiency and (*RS*)-MCPP SUR. In addition, gradual acclimatisation of the biomass with time may have also contributed to the improved average removal and SUR observed with these temporal changes in COD/SO_4^{-2} ratios.

COD/SO_4^{-2} ratio experiment also provides a better understanding of competition between sulphidogenesis and methanogenesis and showed that these microbial groups can be maintained concomitantly in the same reactor and in the presence of (*RS*)-MCPP.

For all COD/SO_4^{-2} ratios, the total COD removal efficiency remained high with minimum value of 70 %, with an increasing proportion of this COD removal (1 to 75 % at ratios of 2 to 0.2, respectively) being linked directly to sulphate reduction.

10.2.4 Effect of HRT on (*RS*)-MCPP degradation under methanogenic conditions - Chapter 8

Improved treatment of wastewater containing (*RS*)-MCPP could be achieved by increasing the operating HRT of the AMBr. (*RS*)-MCPP utilisation rates increased with increases in HRT achieved by decreasing the flow rate. The highest (*RS*)-MCPP SUR (43 $\mu g.mgVSS^{-1}.d^{-1}$) was achieved at 16.9 d HRT when operating at OLR of 0.13 $kgCOD.m^{3-}d^{-1}$. Longer contact time, diauxic growth and probable changes in redox potential arising from different OLR and HRT (as discussed in Section 8.3.5) are the most likely explanation for this observation.

High (*RS*)-MCPP degradation and utilisation rates observed at long HRT (16.9 days) in Phase IV were not caused by aerobic degradation (there was a small chance that traces of oxygen may have entered the reactor by permeate recycling during this period, see Section 8.4) because when the chance of oxygen ingress was completely eliminated in Phase V, no reduction in (*RS*)-MCPP removal efficiency was observed.

When considering reactor performance, the AMBr proved to be stable to large transient hydraulic shock loads, and it recovered rapidly to baseline performance after the hydraulic shock load had ended. Among the difficulties encountered with the AMBr operating at low OLR (high HRT) was the loss of more than 50% of methane produced within the system by solubility in the effluent.

10.2.5 Effect of different redox conditions on (*RS*)-MCPP degradation and the bacterial ecology of the AMBr– Chapter 9

The composition of archael and bacterial group distribution was influenced by different terminal electron acceptors (redox conditions) used in different phases of this research, with methanogenic archaea dominating the microbial community under methanogenic conditions and eubacteria dominating under anoxic conditions. Consistently, *Methanosaeta* was the predominant methanogenic archael species irrespective of redox environment, (*RS*)-MCPP concentrations or HRT.

A comparison of (*RS*)-MCPP utilisation rates in each phase of the research revealed wide ranges (*RS*)-MCPP degradation efficiency and (*RS*)-MCPP SUR, however, although changes were observed in the composition of the bacterial population, no direct linkage could be made with any of the individual groups identified by FISH. Future use of more specific techniques, such as DGGE, may reveal the bacterial group(s) for (*RS*)-MCPP degradation.

Inhibition by (*RS*)-MCPP was only observed in the methanogenic phase, and only at a (*RS*)-MCPP concentration of 200 $mg.L^{-1}$, i.e. no inhibition was observed under anoxic conditions. However, as the methane yields were near to the theoretical values when (*RS*)-MCPP was present at 200 $mg.L^{-1}$ under methanogenic conditions, it was concluded that the methanogens were not inhibited by this level of (*RS*)-MCPP whilst other bacteria, such as fermentative bacteria were partially inhibited, as indicated by slight reduction in overall COD removal efficiency.

10.3 RECOMMENDATIONS

This work was limited in the context of time and resources and further work is required to explore other systems that may yield more effective treatment of (*RS*)-MCPP wastewater under anaerobic conditions, therefore, the following work is recommended:

i. The treatment of (*RS*)-MCPP could be investigated at thermophilic temperature, as (*RS*)-MCPP may degrade faster at higher temperatures therefore enhance overall reactor performance;

ii. To provide a clearer view of which parameter had the greatest impact on (*RS*)-MCPP utilisation rates, OLR and HRT should be investigated separately by varying each parameter independently over a range of values;

iii. To give greater information on the effect of redox conditions, rather than the effect terminal electron acceptors, experiments should be carried out with direct measurement of the oxidation reduction potential (ORP);

iv. Further investigation using molecular techniques into bacterial community structure within the AMBr reactor sludge should be carried out, in particular the cells which are responsible for the degradation of (*RS*)-MCPP under nitrate-reducing conditions (Phase II) and during high operating HRT (Phase IV) should be identified;

v. For optimisation of (*RS*)-MCPP degradation under nitrate-reducing conditions (Phase II), further investigation should be carried out into the operating factors that might increase process performance such as HRT, OLR, trace metal nutrient and pH etc;

vi. Further detailed investigation should be conducted on the biodegradation pathway of (*RS*)-MCPP during AMBr operation. Transformation of the (*RS*)-MCPP during biodegradation by detecting the metabolites within the pathway would help give a clearer overall view of biodegradation process and intermediates could be tested as substrates directly on the microbial populations to identify key species;

vii. The effect of different enantiomers of (*R*)-MCPP should be evaluated since it might have different biodegradation and inhibition rates. For this, separate dosing of reactors with either (*R*)-MCPP or (*S*)-MCPP should be carried out and effect the standard reactor performance indicators assessed.

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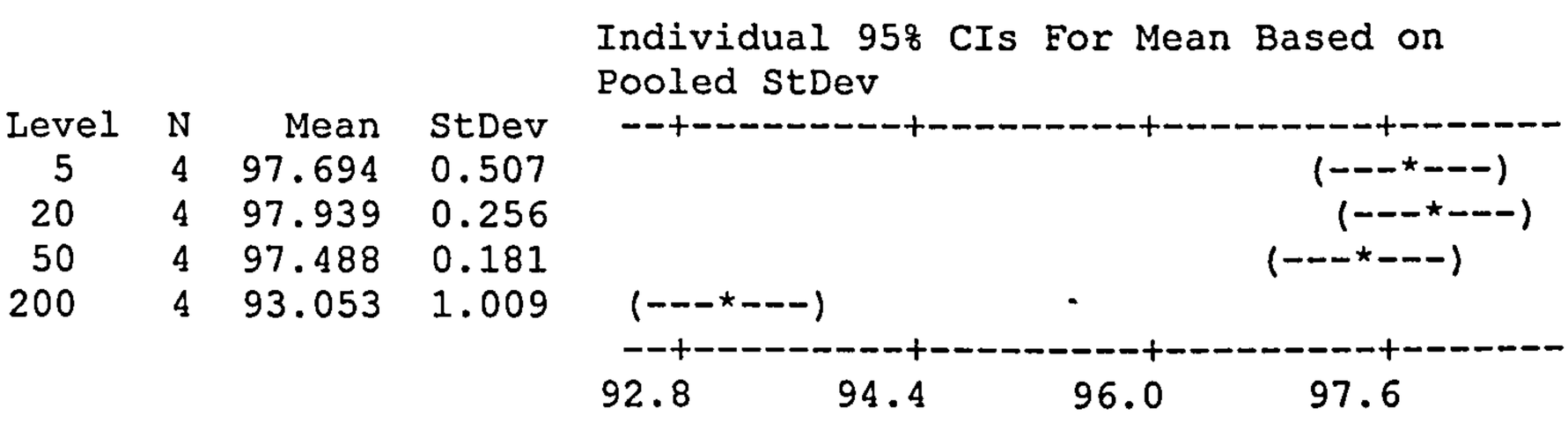
APPENDIX I – Statistical Analysis of AMBr Performance Data

Data analysis was performance by MINITAB V 14 (Minitab Inc., Philadelphia, USA) using the analysis of variance (ANOVA). MINITAB gives the following outputs for the analysis of variances.

One-way ANOVA: %COD Removal versus (*RS*)-MCPP concentration (Phase I)

```
Source      DF       SS       MS       F      P
(RS)-MCPP    3   65.406   21.802   63.51  0.000
Error       12    4.119    0.343
Total       15   69.525

S = 0.5859   R-Sq = 94.08%   R-Sq(adj) = 92.59%
```

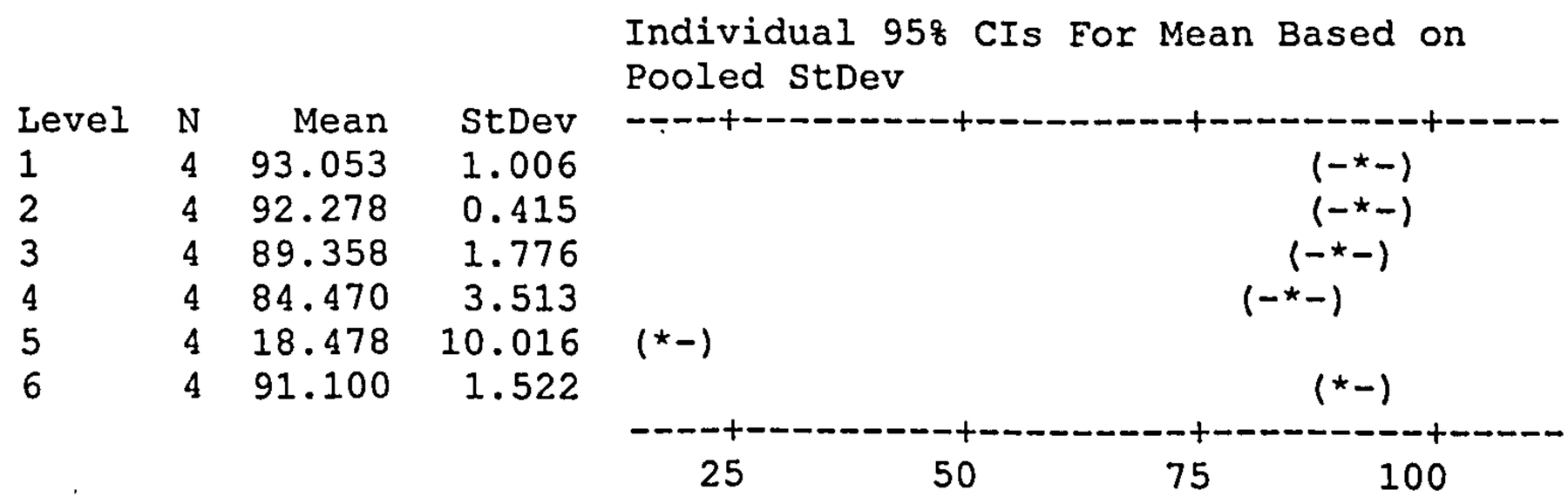



```
Pooled StDev = 0.586
```

One-way ANOVA: %COD Removal versus COD/N-NO_3^- (Phase II)

```
Source  DF       SS       MS       F      P
C2       5  17262.9   3452.6  173.63  0.000
Error   18    357.9     19.9
Total   23  17620.8

S = 4.459   R-Sq = 97.97%   R-Sq(adj) = 97.40%
```




```
Pooled StDev = 4.459
```

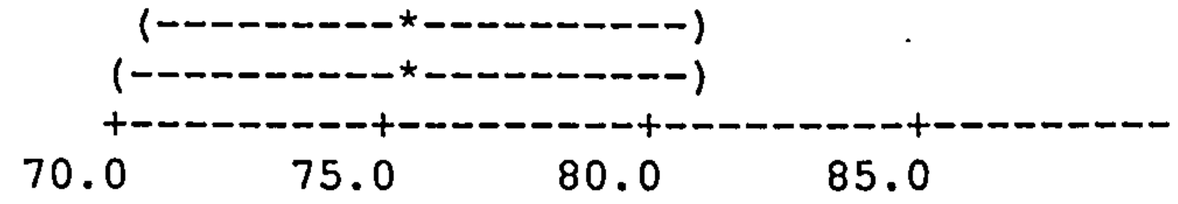
One-way ANOVA: %COD Removal versus COD/S-SO_4^{-2} (Phase III)

Source	DF	SS	MS	F	P
C2	2	217.6	108.8	4.88	0.037
Error	9	200.6	22.3		
Total	11	418.2			

S = 4.721 R-Sq = 52.03% R-Sq(adj) = 41.37%

Level	N	Mean	StDev
1	4	84.588	7.344
2	4	75.630	1.906
3	4	75.480	3.052

Individual 95% CIs For Mean Based on Pooled StDev



Pooled StDev = 4.721

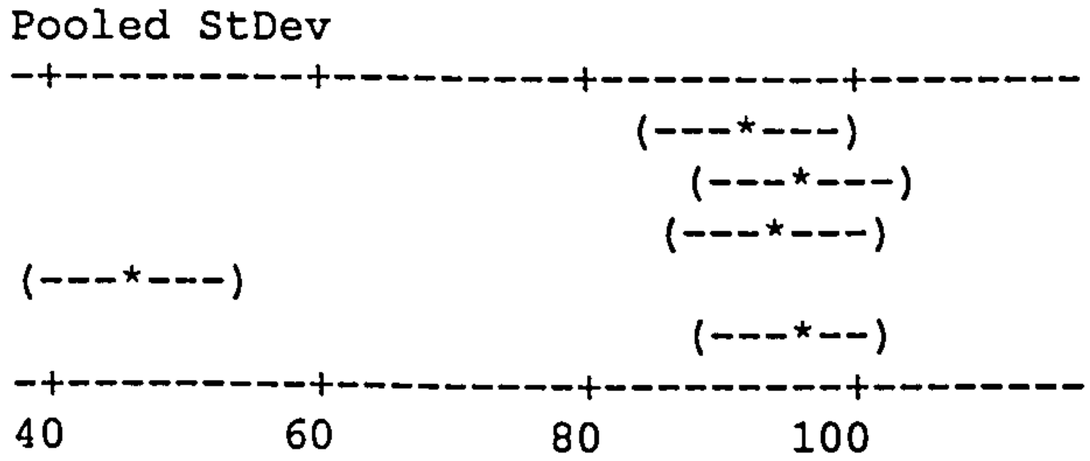
One-way ANOVA: %COD Removal versus HRT (Phase IV)

Source	DF	SS	MS	F	P
C2	4	7480.3	1870.1	35.38	0.000
Error	15	792.9	52.9		
Total	19	8273.2			

S = 7.271 R-Sq = 90.42% R-Sq(adj) = 87.86%

Level	N	Mean	StDev
1	4	92.74	2.88
2	4	96.03	1.10
3	4	94.34	1.97
4	4	46.29	15.83
5	4	95.12	0.59

Individual 95% CIs For Mean Based on Pooled StDev



Pooled StDev = 7.27

Table A1

(*RS*)-MCPP	Phase I	
	Mean ±SD	P(F)
5 mgL^{-1}	97.694±0.507	0.000(63.51)
20 mgL^{-1}	97.939±0.256	
50 mgL^{-1}	97.488±0.181	
200 mgL^{-1}	93.053±1.009	
COD/N-NO_3^-	**Phase II**	
	Mean ±SD	P(F)
250	93.053±1.006	0.000(173.63)
8	92.278±0.415	
3	89.358±1.776	
1	84.470±3.513	
0.3	18.478±10.016	
0.2	91.10±1.522	
COD/S-SO_4^{-2}	**Phase III**	
	Mean ±SD	P(F)
2	84.588±7.344	0.037(4.88)
0.4	75.630±1.906	
0.2	75.480±3.052	
HRT	**Phase IV**	
3.4	92.74±2.88	0.000(35.38)
6.8	96.03±1.10	
16.9	94.34±1.97	
0.8	96.29±15.83	
3.4	95.12±0.59	

SD is an abbreviation of standard deviation. P is the probability calculated for the variances existing across different (*RS*)-MCPP concentrations, COD/N-NO_3^-, COD/S-SO_4^{-2}, and HRT. F is the Fisher value calculated for the variances existing between (*RS*)-MCPP concentrations, COD/N-NO_3^-, COD/S-SO_4^{-2}, and HRT

P= 0.000 means P < 0.001.

APPENDIX II- An Example of Cell Counting and Statistical Analysis

Total Cell Count (DAPI)

Total bacterial count were taken from reactor sludge samples at each phase by using epifluorescent miscroscopy. The number cell per field of view (FOV) was determined for 20 random observations. An example of DAPI counting is given in Table A2 shows mean number of cells per ml in each condition of AMBr by DAPI counting.

Statistical Analysis for DAPI

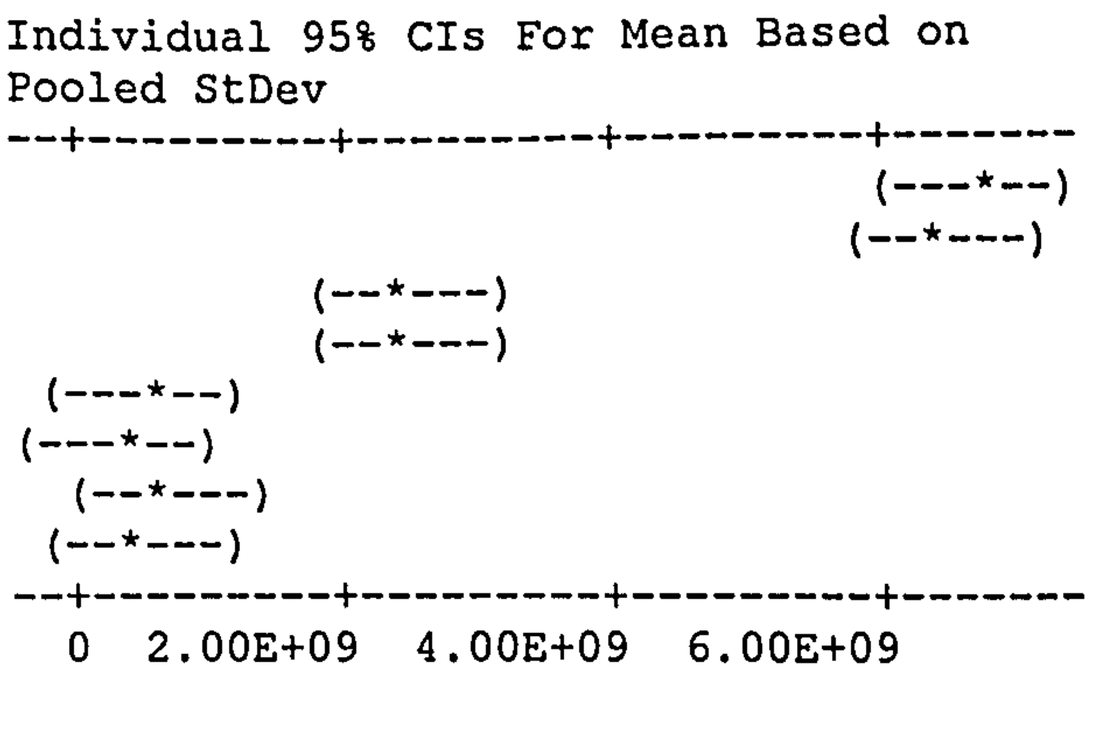
An example of MINITAB output for one way ANOVA of DAPI counting is given below:

One-way ANOVA: Total Cell Count versus Each phase

Source	DF	SS	MS	F	P
factor	7	9.93984E+20	1.41998E+20	55.31	0.000
Error	152	3.90255E+20	2.56746E+18		
Total	159	1.38424E+21			

S = 1602330991 R-Sq = 71.81% R-Sq(adj) = 70.51%

Level	N	Mean	StDev
1	20	6747533333	3780449932
2	20	6419466667	1555668174
3	20	2425033333	1376409185
4	20	2442766667	1235812724
5	20	520473333	401466745
6	20	332056667	155655231
7	20	610470000	400890964
8	20	475696667	244864607



Pooled StDev = 160233099

Table A2

Number of Observations	Phase I		Phase II		Phase III		Phase IV	
	5 mgL^{-1}	200 mgL^{-1}	COD/N 1	COD/N 0.2	COD/S 2	COD/S 0.2	HRT 3.4d	HRT 6.9d
1	59	59	19	16	75	102	110	71
2	164	81	25	14	46	71	82	25
3	38	63	27	13	73	48	67	22
4	22	126	28	9	34	11	74	26
5	43	65	40	32	54	38	79	52
6	60	65	16	38	64	29	127	46
7	55	58	17	45	81	104	194	56
8	77	72	49	16	21	79	81	27
9	49	67	52	40	21	46	52	44
10	43	79	46	11	43	55	21	56
11	62	54	11	43	26	52	71	130
12	141	74	9	45	25	91	47	50
13	38	97	8	18	31	110	15	35
14	43	77	16	21	34	67	24	34
15	111	69	5	26	33	141	46	102
16	107	52	33	44	60	70	21	64
17	128	48	33	32	47	111	44	44
18	93	84	40	52	50	116	137	33
19	41	83	18	24	154	116	32	78
20	148	75	55	12	202	41	53	78
Average	76.1	72.40	27.350	27.55	58.7	75	69	53.65
Total Cell Count	$6.7X10^9$	$6.4X10^9$	$2.4X10^9$	$2.4X10^8$	$5.2X10^9$	$3.3X10^8$	$6.1X10^8$	475696666.7
Log	9.83	9.81	9.39	8.39	8.58	8.52	8.79	8.68

Total cell count for Phase III (COD:S=0.2)

$$\text{Total number of cells per ml} = \frac{\text{Mean (or median) number of cells per FOV} \times \text{Total area of filter (mm}^2\text{)}}{\text{Area of FOV (mm}^2\text{)} \times \text{Volume of sample applied (0.03 ml)} \times \text{Dilutions}}$$

$$3.3X10^8 = \frac{75 \times 132.73}{0.01 \times 0.03 \times 0.01}$$

Cell counting (FISH) for one specific probe and percentage calculation relative to the total DAPI-stained cells

The number of cells for each specific probe was determined and means were calculated for 10 randomly chosen FOV for each sample. This data was used to calculate percentages of one specific group cells, relative to the total DAPI-stained cells. Table A3 and A4 shows the cell count for probe EUB338 and ARC915 in Phase I at (*RS*)-MCPP concentration of 200 $mg.L^{-1}$.

Table A3

Number of observations	Phase I (200mgL^{-1} of (*RS*)-MCPP)	
	EUB338	ARC915
1	27	202
2	25	178
3	58	204
4	54	258
5	43	240
6	98	338
7	110	273
8	84	550
9	250	250
10	25	250
Average Cells	86.0	304.8
Number of Cells per mL	$1.2x10^9$	$4.2x10^9$

Average number of cells (archae) in one ml of sample was calculated using the following equation:

$$\text{Mean number of cells per ml} = \frac{\text{Mean number of cells per FOV} \times \text{Area of sample spot (mm}^2\text{)}}{\text{Area of FOV (mm}^2\text{)} \times \text{Volume of sample applied (0.01 ml)} \times \text{Dilutions}}$$

$$4.2x10^9 = \frac{304.8 \text{ x } 19.63}{0.0141729 \text{ x } 0.01 \text{ x } (0.5 \text{ x } 0.1 \text{ x } 0.2)}$$

Percentage calculations relative to total DAPI-stained cells in Phase I at (*RS*)-MCPP concentration of 200 mg.L^{-1}:

i) EUB338

Number of EUB338 cells = 1.2×10^9

Number of total DAPI-stained cells in Phase I at (*RS*)-MCPP concentration of 200 mgL^{-1} = 6.4×10^9(from Table A2)

Percentage calculations = $(1.2 \times 10^9 / 6.4 \times 10^9) \times 100\ \%$

$= 18.75\ \%$

ii) ARC915

Number of ARC915 cells = 4.2×10^9

Number of total DAPI-stained cells in Phase I at (*RS*)-MCPP concentration of 200 $mg.L^{-1} = 6.4 \times 10^9$ (from Table A2).

Percentage calculations = $(4.2 \times 10^9 / 6.4 \times 10^9) \times 100\ \%$

$= 65.63\ \%$