BIOFLOCCULATION OF ACTIVATED SLUDGE IN RELATION TO CALCIUM ION CONCENTRATION

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

AYSUN VATANSEVER

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
DEPARTMENT OF ENVIRONMENTAL ENGINEERING

JULY 2005

Approval of the Graduate School of Natural and Applied Sciences

Prof. Dr. Canan Özgen
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Filiz B. Dilek
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Assoc. Prof. Dr. F. Dilek Sanin
Supervisor

Examining Committee Members

Prof. Celal F. Gökçay	(METU, ENVE)	
Assoc. Prof. Dr. F. Dilek Sanin	(METU, ENVE)	
Assoc. Prof. Dr. Göksel N. Demirer	(METU, ENVE)	
Assoc. Prof. Dr. Pınar Çalık	(METU, CHE)	
Assist. Prof. Dr. Ayşegül Aksoy	(METU, ENVE)	

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name :

Signature :

ABSTRACT

BIOFLOCCULATION OF ACTIVATED SLUDGE IN RELATION TO CALCIUM ION CONCENTRATION

Vatansever, Aysun

M.Sc., Department of Environmental Engineering

Supervisor: Assoc. Prof. Dr. F. Dilek Sanin

July 2005, 134 pages

Bioflocculation, which can be defined as aggregation of bacterial flocs, has important implications on the physical characteristics of sludge. It is especially critical to settling and dewatering systems which impacts the overall economy of the process greatly. One of the most common problems in activated sludge systems to negatively influence the settleability is sludge bulking which can be defined as non-settling situation of microbial mass.

The first objective of this research is to investigate the effect of calcium ion on sludge bulking in a phosphorus deficient medium and the second objective is to improve the settling, dewatering, and pumping of activated sludge by adjusting the calcium (Ca) ion concentration of the feed. For this purpose, 7 semi-continuous laboratory scale activated sludge reactors were operated with a mixed aerobic culture. The reactors had 8 days of sludge residence time and aerated with air pumps to provide a dissolved oxygen concentration of at least 3 mg/L. All the analyses were conducted after the reactors reached steady state condition.

In the first part of the research, the effect of strictly phosphorus-limited medium on bulking of activated sludge was studied at different calcium ion concentration. Three reactors were set up having 5, 10 and 20 meq/L calcium concentrations. From the results it was observed that, phosphorus deficiency caused viscous bulking independent from the calcium ion concentration. It was found out that bulking of activated sludges due to phosphorus deficiency could be cured by the addition of phosphorus. Furhermore, microorganisms starved for phosphorus, seemed to accumulate polyphosphate granules when they were exposed to a phosphorus source.

In the second part of the study, the effect of calcium ion on physical, chemical and surface chemical properties of activated sludge was investigated at 4 different concentrations (0.27, 5, 10 and 20 meq/L) under sufficient phosphorus concentration. Calcium addition resulted in significant changes in the quantity and quality of extracellular polymeric substances (EPS). Total EPS increased depending on the calcium concentration. Carbohydrate content of EPS dominated over the protein content for calcium concentration of 5 meq/L and above. The amount of calcium ions incorporated into the sludge floc matrix also increased with the dose of calcium added. Settleability and dewaterability of sludge improved significantly at 5 meq/L dose of calcium. However, settleability did not change any further with increasing calcium dose, whereas dewaterability increased for all increasing calcium concentrations. Sludge viscosity also decreased considerably at 5 meq/L concentration indicating better pumpability but it did not change further above 10 meq/L calcium addition.

Key words: activated sludge, extracellular polymers, sludge bulking, settleability, dewaterability, viscosity.

ÖZ

AKTİF ÇAMUR YUMAKLAŞMASINDA KALSİYUM İYONU KONSANTRASYONUNUN ETKİSİ

Vatansever, Aysun
Yüksek Lisans, Çevre Mühendisliği Bölümü
Tez Danışmanı: Doç. Dr. F. Dilek Sanin

Temmuz 2005, 134 sayfa

Bakteri kümelerinin bir araya gelmesiyle oluşan aktif çamur yumaklaşması çamurun fiziksel özelliklerinin belirlenmesinde önemli rol oynamaktadır. Yumaklaşmanın tam olarak gerçekleşememesi, toplam arıtım maliyetini önemli ölçüde etkileyen çökelme ve susuzlaştırma sistemlerinde ciddi sorunlar meydana getirerek arıtma veriminin azalmasına neden olmaktadır. Aktif çamur sistemlerinde çökelebilirliği etkileyen temel sorunlardan biri ise mikroorganizmaların çökmek için yeterli yoğunluğa ulaşamaması olarak tanımlanan çamur şişmesi durumudur.

Bu araştırmanın amaçlarından ilki kalsiyum iyonunun fosforca yetersiz ortamda çamur şişmesi üzerine olan etkisini araştırmak, diğeri ise giriş suyundaki kalsiyum iyonu konsantrasyonunu ayarlayarak çamurun çökelebilme, susuzlaştırılabilme ve pompalanabilme gibi özelliklerinin iyileştirilmesidir. Bu amaçla, 7 yarı-sürekli laboratuar ölçekli aktif çamur reaktörü karışık kültür mikroorganizmalar kullanılarak çalıştırılmıştır. Reaktörlerin çamur alıkonma süresi 8 gün olup hava pompaları ile

çözünmüş oksijen konsantrasyonu en az 3 mg/L olacak şekilde havalandırılmışlardır. Bütün analizler reaktörler kararlı denge durumuna ulaştıktan sonra yapılmıştır.

Araştırmanın ilk kısmında fosfor yetersizliğinin çamur şişmesine olan etkisi kalsiyum iyonu konsantrasyonuna bağlı olarak incelenmiştir. Kalsiyum konsantrasyonları 5, 10 ve 20 meq/L olacak şekilde 3 ayrı reaktör çalıştırılmıştır. Sonuçlar incelendiğinde, fosfor yetersizliğinin kalsiyum iyonu varlığında kalsiyum iyonu konsantrasyonundan bağımsız bir şekilde viskoz çamur şişmesine neden olduğu gözlenmiştir. Fosfor yetersizliği sonucu oluşan çamur şişmesi probleminin fosfor eklenerek giderilebileceği bulunmuştur. Ayrıca fosforca yetersiz durumdan fosforca yeterli duruma geçildiğinde mikroorganizmaların polifosfat granülleri biriktirdiği görülmüştür.

Çalışmanın ikinci bölümünde ise kalsiyumun çamurun fiziksel, kimyasal ve yüzey kimyasal özelliklerine olan etkileri 4 ayrı konsantrasyonda (0.27, 5, 10 and 20 meq/L) araştırılmıştır. Kalsiyum eklenmesi hücre dışı polimerlerin (HDP) nicelik ve niteliğinde önemli değişikliklere sebep olmuştur. Toplam HDP kalsiyum konsantrasyonuna bağlı olarak artmış ve 5 meq/L ve üzerindeki konsantrasyonlarda HDP'deki karbonhidrat miktarı protein miktarından her zaman daha fazla olmuştur. Çamurun yumak yapısına giren kalsiyum iyonu miktarı da artan kalsiyum dozuna bağlı olarak artmıştır. Çamurun çökelebilirliği ve susuzlaştırılabilirliği 5 meq/L kalsiyum dozu ile birlikte önemli oranda iyileşmiştir. Bununla birlikte, çökelebilirlik daha fazla kalsiyum artışı ile değişmemiş susuzlaştırılabilme ise kalsiyum artışına bağlı olarak bütün konsantrasyonlarda gelişme göstermiştir. Çamurun viskozitesi de 5 meq/L'ye geçildiğinde önemli ölçüde azalmış olup pompalanabilirliğin iyileştirildiğini göstermektedir fakat 10 meq/L kalsiyum dozunun üzerinde daha fazla azalma saptanmamıştır.

Anahtar kelimeler: aktif çamur, hücre dışı polimer, çamur şişmesi, çökelebilirlik, susuzlaştırılabilme, viskozite.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor Assoc. Prof. Dr. F. Dilek Sanin for her valuable guidance, advice and efforts, as well as her encouragement, empathy and enthusiasm about this work throughout the research period and in the preparation of this thesis. I would like to send my thanks to her for being much more than a supervisor and for giving me a different perspective of life.

I would also like to thank my other committee members Prof. Dr. Celal F. Gökçay, Assoc. Prof. Dr. Göksel N. Demirer, Assoc. Prof. Dr. Pınar Çalık and Assist. Prof. Dr. Ayşegül Aksoy for their precious suggestions and contributions to this study.

The technical assistance of Kemal Demirtaş, Aynur Yıldırım and Ramazan Demir in the laboratory are gratefully acknowledged.

I am thankful to Bilgen Yüncü for her helps during the experiments and for everything she has done.

I am also thankful to Erkan Şahinkaya, Nimet Uzal and Hande Yükseler for their valuable helps and sharing their technical experiences.

My dear lab-partner İpek Turtin, who had the same destiny with me during this study, we have resisted many difficulties together and it has always been a pleasure for me to work with her.

My dear friends; Nihan Moralı, Volkan Çağın, Sevil Canıyılmaz, Burcu Uyuşur, Recep Tuğrul Özdemir, Serkant Fidan, Hale Aylin Çoruh, Özge Yılmaz, and Gülçin Özsoy, I am grateful to all of you for your endless helps, encouragement and patience during this research. Thank you for being near me whenever I needed.

I would also like to acknowledge to Hacettepe University Environmental Engineering and Biology Departments and Gonca Yılmaz for their technical assistance.

My special thanks go to my parents Hayriye and Muhsin Vatansever, whom I dedicate this work to, for believing and trusting in me throughout my life. Although we have spent 6 years apart from each other, it was their love, interest and endless support passing over the distances that gave me the power to attain what I have achieved.

I would like to thank my sisters Aysel, Ayşe and brother Mustafa for sending their loves to me during the period we were away from each other and for their understanding to me.

And finally I would like to thank TÜBİTAK for providing financial support to this research.

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

ABBREVIATIONS

BOD_5	: Biochemical oxygen demand for 5 days
BSA	: Bovine serum albumin
C/N	: Carbon to nitrogen ratio
CER	: Cation exchange resin
COD	: Chemical oxygen demand
COD/N/P	: Chemical oxygen demand to nitrogn to phosphorus ratio
DO	: Dissolved oxygen
EPS	: Extracellular polymeric substances
F/M	: Food to microrgnism ratio
MATH	: Microbial adhesion to hydrocarbons
MLSS	: Mixed liquor suspended solids
MLVSS	: Mixed liquor volatile suspended solids
SRF	: Specific resistance to filtration
SRT	: Sludge residence time
SVI	: Sludge volume index
TKN	: Total kjeldahl nitrogen
VSS	: Volatile suspended solids

CHAPTER 1

INTRODUCTION

1.1. General Information

Activated sludge process is the most widely used biological process for the treatment of municipal wastewaters and is dependent upon two steps. Initially, the constituent organic matter is utilized by the bacterial cells partially for free energy through oxidation and partially for synthesis of new microbial cells. The second step is labeled the 'flocculation phase,' wherein the bacterial cells aggregate into readily settleable masses or flocs (Gulas *et al*., 1979).

The efficiency of activated sludge systems depends not only on the efficiency of biooxidation step, but also effective separation of the treated effluent from the biological solids. Separation results from settling of spontaneously aggregated bacteria into flocs and a subsequent dewatering is necessary to obtain a reduction in sludge volume to facilitate transport and handling, or to minimize the space or energy needed in case of drying or incineration (Jin *et al*., 2004).

Poor separation of activated sludge in secondary clarifiers because of activated sludge bulking and/or foaming can result in serious operational problems. Bulking occurs when aggregates do not compact but instead form loose, low density flocs (Lau *et al*., 1984; Sponza, 2003). Along wih the numerous other factors, the composition of wastewater, high sludge loading and influent wastewater containing insufficient amount of certain nutrients have been found to be the main reasons of sludge bulking (Jenkins *et al*., 1993).

Bioflocculation which can be defined as aggregation of bacterial flocs is of utmost importance for effective separation of microorganisms from the treated effluent. A typical floc is formed by different species of bacteria together with other organisms like protozoa, fungi, filamentous organisms and viruses along with some abiotic suspended material all of which are held together in a polymeric network called the extracellular polymeric substances (EPS).

By definition, EPS are located at or outside the cell surface. Their composition may be controlled by different processes, such as active secretion, shedding of cell surface material, cell lysis, and adsorption from the environment. A modern concept is that EPS allow microorganisms to live continuously at high-cell densities in stable mixed population communities. In other words, the EPS matrix is a medium allowing cooperation and communication among cells in microbial aggregates. Stable, close proximity of the bacteria requires that the cells be held together by the EPS (Laspidou and Rittman, 2002).

Furthermore, it has been suggested that not only is the amount of EPS, but the type and physical characteristics of EPS may influence bioflocculation (Liao *et al*., 2001). However, conflicting results have been reported in the literature regarding the amount of EPS components which are summarized in Table 1.1.

Previous studies revealed that metal ions have the ability to bind extracellular polymers inducing bioflocculation (Forster, 1985b; Higgins and Novak, 1997a,b,c; Jin *et al*., 2003). Among the different types of cations, the role of calcium, which is a very commonly found metal ion in natural waters, has been emphasized by many researchers (Sanin and Vesilind, 1996; Biggs *et al*., 2001). It has been shown that removal of calcium from sludge resulted in deterioration of floc structure and changed the physical properties of sludge significantly (Keiding and Nielsen, 1997; Sanin and Vesilind, 2000).

Table 1.1. Literature data on the quantities of extracted EPS constituents

Carbohydrate	Protein	Type of wastewater	Researchers
1.1 mg/gVSS	7 mg/gVSS	Municipal	Brown and Lester, 1980
0.7 mg/gVSS	8.6 mg/gVSS	Peptone and meat extract	
54 mg/gVSS	9 mg/gVSS	Municipal	Horan and Eccles, 1986
13.98 mg/gSS	11.39 mg/gSS	Municipal	Morgan *et al*., 1990
23.9 mg/gSS	14.07 mg/gSS	Municipal	
47 mg/gVSS	352 mg/gVSS	Municipal	Frolund *et al*., 1996
17.5 mg/gTSS	67.5 mg/gTSS	Bactopeptone	Higgins and Novak, 1997
1.14%	24.2%	Municipal	Dignac *et al*., 1998
27.5 mg/gSS	90.3 mg/gSS	Municipal	Jorand *et al*., 1998
27 mg/gSS	66 mg/gSS	Municipal	
18.4 mg/gSS	62.1 mg/gSS	Brewery	
12.7 mg/gVSS	162 mg/gVSS	Municipal	Bura *et al*., 1998
28.2 mg/gVSS	85 mg/gVSS	Synthetic feed (COD/N/P = 100/5/1)	
40-65 mg/gVSS	50-60 mg/gVSS	Glucose	Shin *et al*., 2001
33.1 mg/gSS	6.4 mg/gSS	Municipal	Hoa *et al*., 2003

On the other hand, Shimizu and Odawara (1985) reported that a decrease in bioflocculation occurred with the addition of calcium. However, in this study, calcium was added to the system in batch mode with short durations and mono culture bacteria were used which is also the case for majority of the other studies. Moreover, for the limited number of researches which added calcium with the growth media and used mixed culture bacteria, the operational conditions in the aeration tanks and the composition of feed waters to the reactors were different from each other. Hence, no consensus has been reached on the effects of calcium on

bioflocculation. In addition, subsequent settling and dewatering characteristics of sludge in relation to calcium ion has not been thoroughly understood yet.

Therefore, the main objective of this research is to investigate the effects of calcium ion concentration on activated sludge properties with mixed culture bacteria and synthetic feed medium representing the situation in actual activated sludge systems. The effects of different calcium concentrations were studied in both phosphorus deficient and phosphorus present conditions. Results of this work are expected to contribute to the understanding of bioflocculation phenomena which is of extreme significance for the wastewater treatment plants.

1.2. Objectives of the Study

The main goal of this study is to comprehend the bioflocculation phenomena in activated sludge systems in relation to the extracellular polymeric substances and calcium ions.

Two main objectives are established to achieve this main goal. In the first part of the study, the aim is to determine the effect of calcium on sludge characteristics in phosphorus deficient reactors.

In the second part of the study, the aim is to examine the effect of calcium ion concentration on the bioflocculation of mixed culture bacteria specifically aiming:

- to determine the changes in chemical composition of sludge (EPS and calcium content in flocs),
- to determine the changes in surface chemical composition of sludge (hydrophobicity and surface charge) and
- to determine the changes in physical properties of sludge (settleability, filterability and rheoloy).

CHAPTER 2

LITERATURE REVIEW

2.1. Activated Sludge Systems

The activated sludge process presently represents the most widespread technology for wastewater purification. Activated sludge plants can be found in different climate conditions-from the tropics to polar regions, from sea level (wastewater treatment plants in ships) to extreme elevations (mountainous hotels). The scale of activated sludge plants ranges from package plants for one family to huge plants serving big metropolises. The activated sludge process was first developed in 1914 by E. Ardern and W. T. Lockett in England who introduced a recycle of suspension formed during the aeration period. This suspension, called *activated sludge* was in fact an active biomass responsible for the improvement of treatment efficiency and process intensity (Wanner, 1994).

2.1.1. Principles of Activated Sludge System

The objective of an activated sludge process is to remove soluble and insoluble organics from a wastewater stream and to convert this material into a flocculant microbial suspension (Benefield and Randall, 1985). In simple terms, the activated sludge process consists of a reactor called the *aeration tank, a settling tank, solids recycle* from the settler to the aeration tank, and a *sludge wasting line*. The aeration tank is a suspended-growth reactor containing microbial aggregates, or *flocs*, of microorganisms termed the activated sludge. The microorganisms consume and oxidize input organic electron donors collectively called the BOD (Rittman and McCarty, 2001).

The activated sludge is maintained in suspension in the reactor through mixing by aeration or other mechanical means. When the slurry of treated wastewater and microbial flocs pass to the settling tank, the flocs are removed from the treated wastewater by settling, and returned to the aeration tank or wasted to control the solids retention time. The clear effluent is discharged to the environment or sent for further treatment. Capturing the flocs in the settler and recycling them back to the reactor are the keys to the activated sludge process, because they lead to a high concentration of microorganisms in the reactor (Rittman and McCarty, 2001).

Relatively early in the development of activated sludge process, it was empirically established that, to achieve 90 percent removal (or more) of municipal wastewater BOD, aeration times of at least 6 to 8 hours were required. Volumetric loadings of 320 to 800 kg/day/1000 m^3 were applied as well as food to microorganisms ratio of around 0.35. As this treatment approach and efficiency is very popular, the respective process modification is commonly called 'conventional,' and other modifications are usually compared with it. This system is often associated with the plug flow configuration of aeration tanks (length to width ratio of 5 to 50) (Ganczarczyk, 1983).

2.1.2. Microbiology of Activated Sludge Systems

Two crucial characteristics define the kinds of microorganisms in activated sludge. First, the activated sludge contains a wide variety of microorganisms. Prokaryotes (bacteria) and eukaryotes (protozoa, crustacean, nematodes, and rotifers) generally are present, and bacteriophage, which are bacterial viruses, probably reside in the sludge, too. Fungi seldom are important members of the community. Second, most of them are held together within flocs by naturally produced organic polymers and electrostatic forces (Rittman and McCarty, 2001).

The primary consumers of the organic wastes in activated sludge are the heterotrophic bacteria, although with certain organic particles, protozoa may be involved as well. By mass, the dominant members of this community are heterotrophic bacteria (Pike and Curds, 1971). Some bacterial species can consume a

variety of different organic compounds, and others are more specialized, consuming only a small fraction of the organic species present (Rittman and McCarty, 2001).

The bacteria in the activated sludge process include members of the genera *Pseudomonas*, *Zooglea*, *Achromobacter*, *Flavobacterium*, *Nocardia*, *Bdellovibro*, *Mycobacterium*, and the two nitrifying bacteria, *Nitrosomonas* and *Nitrobacter*. Additionally, various filamentous forms, such as, Sphaerotilus, Beggiatoa, Thiothrix, Lecicothrix, and Geotrichum, may also be present (Hawkes, 1963; Higgins and Burns, 1975; Tchobanoglous and Burton, 1991). Besides their role of biooxidation, bacteria are also known for their floc forming abilities which makes their surface and attachment characteristics very important.

The finding about protozoa and other higher life forms indicated that they constitute approximately 5% of the activated sludge biomass and are represented by about 200 species (Curds, 1973; Curds, 1975). Total numbers range from 100 to >100,000/mL. They are usually aerobic and are bacteriovirous (they eat bacteria) and generally dominated by protozoa, with 500 to several thousand/mL observed commonly. These organisms perform several important functions in activated sludge, the most important of which is their removal of nonflocculated bacteria from wastewater through their feeding activities, yielding a clarified effluent (Curds *et al*., 1968; Curds and Fey, 1969). Additionally these organisms may contribute to biomass flocculation through production of fecal pellets and mucus (Curds, 1975) and may function to break up large floc masses and encourage more active biomass through their motility (Javornicky and Prokesova, 1963).

2.2. History of Bioflocculation

Bioflocculation, that is, aggregation and separation of the newly formed biomass from the treated effluent is believed to be primarily as a result of the adhesion of particles by gelatinuous material excreted by bacteria which forms a polymeric network that holds everything together causing the particles to coalesce.

Since the beginning of research into biological waste treatment, bacterial flocculation

has received a great deal of attention. Early investigators (Butterfield, 1935; Heukelekian and Littman, 1939), concerned with the treatment of domestic sewage, noticed the prevalence of a floc-forming organism known as *Zooglea ramigera*. Later studies by McKinney and Horwood (1952) and McKinney and Weichlein (1953) demonstrated that many other bacteria, isolated from sewage sludges, were capable of forming flocs as well (Ganczarczyk, 1983).

McKinney (1952) has proposed a theory of floc formation in which capsulated bacteria are flocculated by direct chemical interactions between adjacent cells. Adsorption of cations from various salts in solution results in a reduced net surface charge which, under the influence of agitation, allows the cells to approach one another very closely. Unfortunately, McKinney's emphases on capsulated bacteria and later discoveries that non-capsulated bacteria demonstrate clear floc forming abilities have led some authors to reject this approach to bioflocculation (Pavoni *et al*., 1972). Later on, however, support was given by Tezuka (1969) to this theory. While working with a non-capsulated Flavobacterium, negatively charged surfaces of adjacent cells were found to be bridged by ionic bonds intermediated by calcium and magnesium in accordance with the theory developed by McKinney. Paradoxically, Tezuka (1967) had observed redispersion of the suspension of Zooglea or Pseudomonas with addition of magnesium salt (Ganczarczyk, 1983).

Furthermore, Tenney and Stumn (1965) have proposed that flocculation of microorganisms was also affected by an interaction of polymers excreted by microorganisms. Then extracellular material was isolated from cell-free supernatant of a well flocculated activated sludge culture and it was observed that polymers of bacterial origin was also capable of destabilizing dispersions of bacteria and abiotic sols similar to synthetic polyelectrolytes (Busch and Stumn, 1968).

Several studies were performed to demonstrate the polyelectrolyte nature of chemically induced bacterial coagulation using synthetic anionic and nonionic polyelectrolytes. It was shown that reduction of surface potential is not a prerequisite for flocculation; agglomeration apparently results from the specific adsorption of the polymers segments and from bridging of polymers between cells (Tenney and

Stumn, 1965; Busch and Stumn, 1968). Hence, although bacterial surface charge plays a very significant role in agglutination mechanisms, it can not be regarded as the predominant reaction controlling the bioflocculation (Pavoni *et al*., 1972).

It is now accepted that bioflocculation occurs via extracellular polymeric substances either produced by the microorganisms or contributed by wastewater (Friedman *et al*., 1969; Pavoni *et al*., 1972; Horan and Eccles, 1986; Urbain *et al*., 1993). The analysis of EPS originating from the microbial system and their interactions with calcium ions is the subject of this research that will be delineated with details in the further sections.

2.3. Extracellular Polymeric Substances (EPS)

Extracellular polymeric substances (EPS) can be described as being high molecular weight compounds (M_w>10,000) produced by microorganisms under certain environmental conditions. Such biopolymers originate from biological synthesis, adsorption of organic matter (cellulose, humic acids, etc.), metabolic excretion or as lysis products from the lytic activity, predominantly of bacteria, and are usually found on floc surfaces (Goodwin and Forster, 1985; Morgan *et al.,* 1990; Urbain *et al*, 1993, Frolund *et al*., 1996). Li and Ganczarczyk (1990) found that EPS form the third biggest component in an activated sludge floc after the cells and water.

Extracellular polymeric substances help bacteria to attach surfaces and protect the cells from phagocytosis, predation, desiccation and endotoxins. They act as reserves of carbon and energy source. They also play a role in metal complexation aiding uptake of cations and dispersals particularly in flocculation (Wilkinson, 1958). Extracellular polymers are subdivided EPS into two categories; as *bound EPS* (sheaths, capsular polymers, condensed gel, loosely bound polymers, and attached organic material) and *soluble EPS* (soluble macro-molecules, colloids, and slimes) (Gehr and Henry, 1983; Hsieh *et al*., 1994; Nielsen *et al*., 1997; Laspidou and Rittman, 2002). The second type is probably more accessible to extraction. The low extraction effciency indicates that a large proportion of the EPS is firmly bound (Wilen *et al*., 2003b).

2.3.1. Composition of Extracellular Polymeric Substances (EPS)

Extraction and analysis of EPS showed that it is composed of mainly proteins, carbohydarates, nucleic acids (Friedman *et al.,* 1969; Pavoni *et al*., 1972; Urbain *et al*., 1993; Jorand *et al*., 1995; Bura *et al*., 1998), lipids (Goodwin and Forster, 1985) humic substances (Dewalle and Chian, 1974; Frolund *et al*., 1996, Wilen *et al*., 2003b) and heteropolymers such as glycoproteins (Horan and Eccles, 1986; Jorand *et al*., 1998).

Analyses of these polymers showed contradictory results regarding the dominant type of polymer in EPS structure. Some researchers identified the polysaccharides as the predominant constituent of EPS (Friedman *et al.,* 1969; Pavoni *et al*., 1972; Norberg and Enfors, 1982; Goodwin and Forster, 1985, Horan and Eccles (1986); Morgan *et al*., 1990; Bejar *et al*., 1998) whereas the others reported that exocellular protein concentration is greater than exocellular polysaccharide concentration (Tenney and Verhoff**,** 1973; Brown and Lester, 1980; Barber and Veenstra, 1986; Eriksson and Alm, 1991; Urbain *et al*., 1993; Higgins *et al*., 1997a,b; Jorand *et al*., 1998; Bura *et al*., 1998; Wilen *et al*., 2003b).

The polymers produced by activated sludge microorganisms under quite different conditions (intensive growth, intensive starvation or decomposition) contained the same sugars (rhamnose, fucose, mannose, galactose, glucose), amino sugars (galactosaminuronic acid, glucosamine, galactosamine) and amino acids (lysine, histidine, argigine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyroine, phenylalanine, and γ-aminobutyric acid). For a complete mix system, the percentages of sugars, aminosugars, uronic acids and amino acids in the volatile fraction of microbial polymers were measured as 28.7%, 10.3% 3.2% and 25.9% respectively (Hejzlar and Chudoba, 1986).

Tenney and Stumn (1965) demonstrated that the isoelectric point of most bacteria are at very low pH values (pH 2-4) and are significantly lower than those of proteins (pH

5-8). This fact suggests the occurrence of substances that have lower isoelectric points than proteins such as glutamic acid, lipids and gluco- and mucopolysaccharides on the external surface of microorganisms. In particular, the later microelectrophoresis studies, by providing data about the isoelectric points and pK values of sludge surfaces, have led to the suggestion that acidic polysaccharides play an important role at the surface of non-bulked sludges (Foster, 1971; Goodwin and Forster, 1985). This has been confirmed by chemical analysis of EPS (Goodwin and Forster, 1985).

Horan and Eccles (1986) detected only five monomers as a result of characterization and comparison of purified exopolysaccharide fractions from five different effluent treatment works namely: glucose, galactose, mannose, glucuronic acid and galacturonic acid. In additiıon, extracellular polymers contained 66% carbohydrates, 8% DNA, 16%RNA and 10% protein.

Hejzlar and Chudoba (1986) isolated the soluble waste products of activated sludge microorganisms grown under different conditions. All isolated polymers contained sugars, amino sugars, uronic acids and amino acids, and biodegradability tests confirmed that the polymers are refractory with the BOD rate constant of 0.03-0.04d^{-1}.

In another study, EPS analysis showed that between 60 and 80% of extracellular substances could be attributed to protein with the remainder consisting of DNA and carbohydrate. (Sponza, 2002). Several classes of proteins exist in the exocellular environment of bacteria. These include extracellular enzymes, proteinaceous S-layers, lectins, intracellular protein from cell lysis or cell-wall turnover, or polypeptide capsular material. The extracellular protein extracted from activated sludge samples could be from a combination of these sources. Of the possible proteins, lectins are one of the most plausible types that could be involved in bioflocculation (Higgins *et al*., 1997c).

Dignac *et al*., (1998) compared the results of different EPS extraction techniques and concluded that about 70 and 80 % of the extracellular organic carbon could be

attributed to proteins and sugars, proteins being the major componenets of EPS. They also commented that the remaining 20 to 30 % uncharacterized organic carbon of EPS are possibly composed of: i. humic compounds, quantified in the EPS by Eriksson and Alm (1991) and Frolund *et al*. (1996) by means of colorimetric methods. Polyphenolic structures (humic and fulvic acids or lignins) found in the flocs are supposed to come from wastewater (Urbain *et al*., 1993); ii. uronic acids, that are specific components of extracellular and cell wall material, but are found in very low concentrations in activated sludge EPS (Frolund *et al*., 1996), or amino sugars, that are the primary units of bacterial cell walls; iii. nucleic acids originating from cell lysis, and accumulated in the EPS. Extracellular nucleic acids were shown to account for 1% and 2% of the VSS of the sludge by Urbain et al. (1993) and Frolund et al. (1996). iv. lipids, identified in EPS by Forster and Clarke (1983) and Goodwin and Forster (1985). The possible occurance of lipids in EPS was investigated and examinations showed that fatty acids coming from mono-di- and triglycerides or phospholipids, and sterols represented less than 1% of the total organic carbon of the EPS extracted (Dignac *et al*., 1998).

Pyrolysis/GC/MS of freeze dried samples was used to characterize the EPS. Proteins being the dominant type of EPS, the main fragments obtained were; pyridine, methylpyridine, styrene, pyrrole, methylpyrrole, benzonitrile, indole and methylindole. Phenol and cresol are known to be produced both by tyrosine and polyhydroxyaromatic compounds (PHA), but the relative proportions of p-cresol and phenol found in EPS were characteristic of tyrosine, which is a para-phenolic amino acid. Only few secondary fragments could be attributed to neutral sugars (furaldehyde or methylfurfural). The presence of nucleic acids in EPS was confirmed by the large furfuryl alcohol peak, while the amino sugar presence was suggested by the acetamide peak. Amino sugars are known to be important constituents of bacterial cell walls that can be released as a result of cell lysis. None of the characteristic peaks of lignins (methoxyphenols) have been characterized in the pyrochromatogram of EPS and very few minor peaks could be attributed to fatty acids (Dignac *et al*., 1998). On the contrary, Higgins and Novak (1997c) revealed the presence of a single protein in the exocellular biopolymer extract from municipal, industrial and laboratory activated sludge samples. Amino acid analysis and amino

acid sequencing results suggested the protein was a lectin-like protein, and binding site inhibition studies demonstrated that the protein had lectin-like activity. Lectins are defined as nonenzymatic proteins that bind sugar residues and play a role in attachment and colonization of bacteria in both animals and plants (Mirelman and Ofek, 1986).

To investigate the EPS composition, laboratory-scale activated sludge reactors were operated under steady-state conditions. Protein was found to be the predominant polymer followed by polysaccharide and DNA. Lower amounts of protein and higher DNA levels were found in the extracellular substances from flocs grown on more complex organics and substrates. A high level of protein (nearly 70 mg g-1 VSS) and a low DNA content (nearly 6mg g-1 VSS) were measured in the EPS of the winery industry and municipal activated sludge while lower levels of protein (approximately 38–42 mg g-1 VSS) and higher levels of DNA content (approximately 11–17 mg g-1 VSS) were measured in the pulp-paper, textile (cotton knit fabrics) and petrochemical floc EPSs under steady-state conditions (Sponza, 2003).

Jorand *et al*. (1998) characterized the EPS of different sludge samples and obtained 90.3 ± 0.4 mg/gSS proteins and 27.5 ± 0.2 mg/gSS carbohydrates for urban sludge, 66 ± 4 mg/gSS proteins and 27 ± 2 mg/gSS carbohydrates for pilot sludge (urban wastewater) and 62.1 ±0.1 mg/gSS proteins and 18.4 ± 0.3 mg/gVSS for brewery sludge.

Morgan *et al*. (1990) on the other hand, measured 69.9 mg EPS/gSS characterized as 13.98 mg carbohydrate/gSS and and 11.39 mg/gSS for lab-grown activated sludge. Furthermore, for a treatment plant activated sludge they identified 90.2 mg EPS/gSS including 23.9 mg carbohydrate/gSS and 14.07 mg protein/gSS.

According to the EPS analysis of sludge samples from Kawada and Tagawa Treatment Plants EPS were composed of protein, RNA, DNA and polysaccharides in all cases. On sludge dry weight basis; proteins constituted 3.65% and 1.29% of EPS, polysaccharides measured as 1.19% and 0.672%, RNA measured as 3.59% and 1.36% and DNA measured as 1.24% and 0.314% respectively (Kakii *et al*., 1989).

Liao *et al*. (2001) reported that the absence of acidic polysaccharides within the EPS is noteworthy. Some studies indicated that acidic polysaccharides are the main components of EPS associated with bacteria (Kenne and Lindberg, 1983; Figueroa and Silverstein, 1989). Acidic polysaccharides, however, were detected (0.5-1.5 mg/g VSS) only during the early stage of the study (Liao *et al*., 2001). These levels were significantly lower than the levels of acidic polysaccharides detected in the inoculum (4.8 mg/g VSS) and in sludge from other full-scale activated sludge systems (Urbain *et al*., 1993; Frolund *et al*., 1994,1996; Bura *et al*., 1998). Acidic polysaccharides were not detectable in the extracted EPS of sludge examined following the stabilization period from the reactors at all SRTs. Jahn *et al*. (1997) have also reported the absence of acidic polysaccharides in the EPS of sludge grown in a chemostat fed a synthetic wastewater (Liao *et al*., 2001).

As it is evident from the literature, the quantity and quality of extracellular polymers exhibits discrepancies from one research to another. The possible reasons of having such various results have been investigated and the findings are discussed in the following section.

2.3.2. Factors Affecting the Production of Extracellular Polymeric Substances

One of the factors that affect EPS production is the growth phase of microorganisms. Previously it was assumed that extracellular polymers were excreted into the cultivation medium at the end of exponential (logarithmic) growth phase and during endogenous growth. (Tenney and Stumn, 1965; Friedman *et al*, 1969; Pavoni *et al*., 1972; Norberg and Enfors, 1982). Remaining carbon at the end of the exponential phase is used for the production of polysaccharides in the stationary phase. In the decay phase, cell lysis takes place. During the early stage of the decay process, there is a sharp increase of polymer production, while later in the decay phase, there is no further production of polymer (Busch and Stumn, 1968; Pavoni *et al*., 1972). However, Gulas *et al*. (1979) obtained reduction in exocellular polymer content per unit of MLVSS as endogenous respiration conditions were approached. A later study indicated that EPS are formed both during growth and during starvation and decomposition of activated sludge microorganisims (Hejzlar and Chudoba, 1986).

Sutherland (1990) later stated that microorganisms differ with respect to the growth phase during which exopolysaccharide is produced. *Xanthomonas campestris* is unusual in that xanathan synthesis occurs throughout growth and into the stationary phase. Other microbial cells may behave very differently indeed. Bacterial alginate synthesis by *Pseudomonas aeroginosa* is mainly during the exponential phase of growth, whereas production of a heteropolysaccharide by another *Pseudomonas* species only started late in the exponential phase and continued to reach a maximum in the stationary phase.

Although little is known about the factors affecting EPS production, there is a general consensus that sludge residence time (SRT) is an important parameter However; contradictory results have been obtained on the effect of SRT on EPS production. Some researchers suggested that total amount of EPS was positively affected by SRT (Chao and Keinath, 1979; Sesay and Sanin 2004) while the others observed that EPS decreased with increase of SRT (Gulas *et al*., 1979; Sheintuch et al., 1986; Wilen *et al*., 2003b). Moreover, some researchers observed that EPS production was independent of SRT. Liao *et. al*. (2001) found that SRT over the range of 4 to 20 days had little effect on the overall EPS, but had a significant effect on protein and carbohydrate components individually. Protein to carbohydrate ratio increased between 4 days and 12 days of SRT and further decreased between 16 and 20 days. A change in the ratio of proteins to carbohydrates in the EPS has been reported to occur in the EPS of biofilms as they age (Jahn and Nielsen, 1996). It is possible that sludge at lower SRTs did not consume all the carbon sources available for growth. Excess carbon substrates could have been converted to intracellular storage granules and extracellular polymers that accumulated as EPS. At higher SRTs with a lower food to microorganism ratio, the level of storage carbohydrate decline which reflected the available carbon (Liao *et al*., 2001). However, Sesay and Sanin (2004) observed that carbohydrate to protein ratio in the polymer decreased between 4 and 20 days of SRT, and explained this with the increase of lytic products such as proteins as the system approaches endogenous growth phase.

Andreadakis (1993) showed that over the sludge age range of 1.1-17.4 days the average carbohydrate content was between 8.5 and 11%. The high sludge

carbohydrate content at high sludge ages (8-14) reflects the accumulation of extracellular polymeric material. At sludge age of 1.1 days the high carbohydrate content was probably caused by accumulation of stored material which resulted in sludge dispersion. The decline in the sludge carbohydrate content observed at a sludge age 17.4 days is striking in view of the expected enhanced exocellular polymer production under starvation or semi-starvation conditions. An explanation was given by considering the ability of the bacteria to acclimatize and produce enzymes which can hydrolyze and assimilate the exocellular polymers surrounding and bonding their surface under conditions of depletion of external substrate (Gaudy *et al*., 1971; Pavoni *et al*., 1972; Yang and Chen, 1977).

Well-controlled laboratory studies have revealed that nutrients (COD/N/P) can influence the composition of the floc matrix and the structure of the floc (Bura *et al*., 1998). Four bench-scale sequencing batch reactors were fed with synthehic wastewater having COD/N/P ratios of 100/5/1, 100/5/0, 100/5/0.2 and 100/1/1. The carbohydrate content of EPS was measured as 28.8, 50, 58.3, 28.1 mg/gVSS and protein amount was measured as 85, 106, 98.8, 20,3 mg/gVSS for the aforementioned COD/N/P ratios, respectively. From these results it is apparent that a significat decline occured in protein content as C/N ratio was increased from 20 to 100. The carbohydrate content on the other hand remained almost the same. Durmaz and Sanin (2001) also reported that when C/N ratio was increased fom 5 to 17.5 and then to 40, the carbohydrate content increased considerably contradicting the results obtained by Bura *et al*. (1998).

Activated sludge systems treating municipal wastewaters mostly operate under carbon limited conditions. The typical municipal wastewater composition has a carbon to nitrogen (C/N) ratio that varies between 25/1 to 20/1; carbon defined in terms of COD and nitrogen defined in terms of ammonium ion (Gaudy and Gaudy, 1980). Allison and Sutherland (1987) reported that when bacteria were grown under glucose limiting conditions only trace amounts of carbohydrate could be detected associated with the attached cells. Harris and Mitchell (1973) discussed that under carbon limited growth, the synthesis of extracellular polymers would be unlikely, however even these microorganisms are known to be able to aggregate. The reason is

supposed to be the excretion of intracellular polymers, or polymers becoming extracellular as the result of partial cellular lysis.

Another reason which causes differences in EPS composition is the type of the microorganisms used. Burdman *et al*. (2000) analyzed the exopolysaccharide and capsular polysaccharide composition of four *Azospirillum brasilense* strains differing in their aggregation capacity. When growing the different strains in an aggregation inducing medium containing a high carbon to nitrogen (C/N) ratio, both exopolysaccharide and capsular polysaccharide showed a positive correlation between aggregation and the relative amounts of arabinose (which is a monosaccharide). However, the relative amounts of arabinose present in the EPS of these four strains when grown in the high C/N medium were significantly different between each other. Similarly, Bejar *et al*. (1998) investigated the chemical composition of exopolysaccharides produced by 19 strains belonging to *Halomonas eurihalina* and found that although the extracted carbohydrate amount was greater than proteins, the amount of carbohydrate was different among the strains. Although it is known that activated sludge systems treating municipal wastewater are comprised of mixed culture bacteria, it is possible that one major group among these bacteria dominate the others and change the composition of EPS.

The polymer extraction method used is another reason of conflicting reports in the literature. Researchers have used different techniques some of which are harsher than the others leading to the lysis of the cells themselves and increase the EPS concentration. Unfortunately, a universally accepted method that will have less effect on whole cell has not been offered yet.

Cation concentration in the wastewater is another factor that influences the amount of extracted EPS. Studies have shown that metal ions have the ability to bind extracellular polymers inducing bioflocculation (Busch and Stumn, 1968; Higgins and Novak, 1997abc; Murthy and Novak, 1998; Sanin and Vesilind, 2000; Li, 2005). However, conflicting results have been obtained regarding the effects of divalent and monovalent cations and the specific affinities of these metals for the specific EPS components which will be discussed in the following sections.

In brief, operational conditions of the aeration tank (sludge residence time, food to microorganisms ratio, C/N ratio), compositions of wastewater and feed media, type of the sludge used in the experiments (treatment plant sludge or lab-grown sludge), type of the microorganisms (pure culture or mixed culture), presence of cations and the extraction method used for the EPS are the factors that influence either the production or the recovery of EPS and explain some reasons of the contradicting findings in the literature.

2.3.3. Physical and Chemical Properties of Extracellular Polymeric Substances

Extracellular polymers which can be in the form of both capsule and slime are gelatinous materials with about 98% water content. The nature, composition and molecular weight of sludge exocellular polymers are of fundamental in determining the settling properties of activated sludges (Horan and Eccles, 1986). Hence, investigations have been performed to understand the physicochemical properties of EPS which constitute the basis of floc formation.

Molecular weight is one of the properties of EPS affecting flocculating ability and so as the settling characteristics significantly. It has been reported that considerable portion of microbial products are high-molecular substances having molecular weights (MW) above 10,000 (Painter, 1973; Parkin and McCarty, 1981; Forster, 1982; Grady *et al*., 1984). Forster (1985a) and Sutherland (1990) suggested that most of the EPS components approximate a molecular weight of about one million. Molecular weight distributions were also obtained with a gel chromatography system whose exclusion limit was four million. These results showed the presence of several high molecular weight (>100,000) species with the highest exceeding $2x10^6$ (Forster, 1985a).

Characterization and comparison of purified exopolysaccharide fractions from five different effluent treatment works revealed many similarities both in terms of monomer composition and molecular weight distribution that all the polysaccharide fractions were of high molecular weight ranging from $3x10^5$ to $2x10^6$ (Horan and Eccles, 1986).

The hydrophilic/hydrophobic interactions of EPS play an important role in the cohesiveness and other properties of bacterial aggregates. EPS was fractioned into its hydrophilic and hydrophobic components using XAD-8 and XAD-4 resins consecutively, an indication that the EPS embrace different polarities. A significant proportion of the EPS fraction (at least 7% in terms of DOC) was observed to be hydrophobic. The hydrophobic fraction was made up of proteins but not carbohydrates (Jorand *et al*., 1998; Liao *et al*., 2001). Electrostatic bonds were found to be uniformly distributed in the floc and closely combined with hydrophobic bonds and mainly amino acids contributed to the hydrophobicity in EPS structure (Bengtsson, 1991; Dignac *et al*., 1998; Liao *et al*., 2001). Alanine, leucine and glycene were the important aminoacids exhibiting hydrophobic properties (Dignac *et al*., 1998)

In the study performed by Wilen *et al*., (2003a) the correlations between the individual EPS and sludge components and hydrophobicity were relatively moderate, thus they stated that other factors or other components in the EPS contribute to the hydrophilic/hydrophobic nature of the sludge. An example to such factors was proposed to be the ratio between the individual components protein, carbohydrate and sum of the organic fractions and the amount of cations in the sludge as it correlated positively to the hydrophobicity. Furthermore, they have also concluded that the more hydrophilic fractions of the polymers are bound to cations.

Since the EPS are a major component of flocs, and have charged functional groups, it is reasonable to assume that they contribute to the net surface properties of the flocs, and thus the flocculation properties (Wilen *et al*., 2003a). It has been found that EPS contribute to the overall negative surface charge of the sludge flocs (Horan and Eccles, 1986; Morgan *et al*., 1990; Keiding and Nielsen, 1997; Mikkelsen and Keiding, 2002). Dignac *et al*., (1998) stated that about 25 % of EPS amino acids were negatively charged and 24% exhibited hydrophobic properties, highlighting the specific role of proteins in the floc structure.

The concentration and nature of the ionogenic polymers present at sludge surface determines the magnitude of the surface charge in the range of -10 to -20 mV. At the upper end of this range it is expected that there will be a sufficiently large floc-repulsion to result in poor sludge settling properties (Forster and Dallas-Newton, 1980).

A relationship between the concentration of surface biopolymers and the sludge surface charge was also established. The correlation coefficient for this relationship was 0.66 which was just significant at the 95% level. A comparative study was made between treatment plant and lab-grown activated sludge samples measuring both the colloidal surface charge of sludge solids and extracted EPS. Lab-grown activated sludge and its extracted EPS had -0.683 and -0.91 miliquivalent/gSS surface charge respectively whereas treatment plant activated sludge and extracted EPS had -0.895 and -0.5 mequivalent/gSS charge on them (Morgan *et al.,* 1990).

The monosaccharide composition and secondary structure of the branched biopolymers help determine the overall surface charge, with the uronic acids playing a major role. Measuring total uronic acids will not create a complete picture of bacterial surface charge, but measuring exopolysaccharide composition will begin to describe the complex exopolysaccharide structure that determines the effective charge on the surface. This effective charge character is the primary determinant for selecting a cationic flocculant, and when supplemented with measures of cell surface hydrophobicity, begins to paint a better picture of floc surfaces (Boyette *et al*., 2001).

Microbial polysaccharides vary considerably in their physical properties, including rheological characteristics. Many of the polymers show pseudoplastic flow (shear thinning), with apparent decrease in viscosity as the shear rate increases. The rheological properties of a polysaccharide can be greatly affected by relatively small changes in chemical structure (Sutherland, 1990).
The rheological properties of the exopolysaccharides produced by 19 strains belonging to *Halomonas eurihalina* in two different culture media. The influence of pH on the viscosity was studied by lowering the pH of the EPS solutions to 3.0. The viscosity of EPS solutions was not particularly high ranging from 15-100 cP at pH 7.

EPS from strain H96 had the best rheological behaviour reaching a viscosity value of 30, 000 cP at pH 3. This gelificant capacity was attributed to its high uronic acid content and it was stated to be attractive for industrial application due to not only its unique composition but also high viscosity of its solution at acidic pH (Bejar *et al.*, 1998).

Analysis of the isolated extracellular polymer revealed that an aqueous solution of this material was capable of imparting a negative electrophoretic mobility to Al_2O_3 particles of incipient positive surface potential at a pH of 7 indicating that the interfacially active components in the cell free supernatant were anionic in the neutral pH range (Busch and Stumn, 1968). Later, Sutherland (1990) discovered that extracellular polymers contain a range of ester linked groups and pyruvate ketals. Ester linked groups did not contribute to the overall charge of macromolecules while pyruvate ketals added to the overall anionic nature of EPS, uronic acids and phosphate groups also contributed to anionic capacity of EPS.

Interaction with cations is another property of extracellular polymeric substances. The adsorption of metal ions occurs on the biopolymers which act as the sludge matrix and whilst these may be polysaccharides, proteins, or even lipids (Forster, 1985b). The presence of cations markedly affects the polymer configuration. For example, in the sodium form, xanthan is a stretched coil, whereas in the calcium form it is a five-fold helix in which the divalent cations are strongly bound to the polysaccharide (Sutherland, 1990).

Metals like Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+} are commonly found in water and wastewater systems and are believed to be complexed by extracellular polymers. Specifically, divalent cations are known to be involved in the chemical structure of bacterial aggregates (Steiner *et al*., 1976; Eriksson and Alm, 1991) because their ability to bind the negatively charged chemical groups (Steiner *et al*., 1976; Urbain *et al*., 1993). The hydroxyl and carboxyl groups are believed to be the sites of metal ion binding on extracellular polymeric structure (Forster, 1985b; Kakii *et al*., 1989; Morgan *et al.*, 1990; Higgins and Novak, 1997a).

Forster and Level (1972) and Forster and Dallas-Newton (1980) detected different affinities between Ca^{2+} and Mg^{2+} ions for extracellular polymers. They found that addition of magnesium ion to sludge has no effect on the bound water content of the sludge, whereas, the bound water was reduced considerably with addition of calcium ions. The ionic size of the cations were also reported to be important for binding ability since the frequency of carboxyl units in the surface polysaccharides increases with ionic size (Foster, 1985b). Thus as calcium ions are larger than magnesium ions (Lide, 1990) their binding may be favoured (Urbain *et al*., 1993).

Similarly, Urbain *et al*., (1993) concluded that exocellular polymers are involved in the formation of a three dimensional matrix or gel where divalent cations, at least Ca and Mg, act as bridging agents with probably specific affinities for each kind of exocellular polymer. They analyzed the physicochemical structure of 16 sludge samples from different origins and observed higher amounts of calcium in EPS than magnesium. These results are all consistent with the findings of other researchers who stated that Ca ions are preferred by extracellular polymers more than Mg ions (Kakii *et al*., 1985; Ericksson and Alm, 1991; Bruus *et al*., 1992; Higgins and Novak, 1997a).Dignac *et al*. (1998) stated that the cationic bonds existing in the flocs concern proteins more than polysaccharides, which was already suggested by the high correlation coefficient found by Urbain *et al*. (1993) and Higgins and Novak, (1997a,b,c).

2.4. Mechanisms of Bioflocculation

A significant amount of research has been performed to better understand the mechanisms of bioflocculation and the solid/liquid separation process. Separation results from the aggregation of microbes into floc particles which can then be separated in part by sedimentation, and further removal of water can be accomplished by gravity thickening and mechanical means. Since particle size is a critical factor in settling and dewatering of activated sludge suspensions, the process and extent of bioflocculation will ultimately determine the settling and dewatering properties. Thus, identification of bioflocculation mechanisms and enhancement of bioflocculation are considered integral to the activated sludge plants.

Various mechanisms of varying complexity for floc formation have been suggested in the literature which are not necessarily mutually exclusive. They can be listed as *Zooglea ramigera* theory (Butterfield, 1935; Heukelekian and Littman, 1939), filament backbone model (Sezgin *et al*., 1978), DLVO type interactions (double layer theory) (Zita and Hermansson, 1994), polymer bridging (Pavoni *et al*., 1972; Harris and Mitcell, 1973), metal ion bridging theory (Busch and Stumn, 1968; Forster and Lewin, 1972; Kakii *et al*., 1985; Eriksson and Alm, 1991; Bruus *et al*., 1992), alginate-like gel formation (Bruus *et al*., 1992; Sanin and Vesilind, 1996; Örmeci and Vesilind, 2000) and hydrophobic interactions (Valin and Sutherland, 1982; Urbain *et al*., 1993; Jorand *et al*., 1998).

The *Zooglea ramigera* theory of bacterial aggregation held that flocculation in the activated sludge process was solely dependent on this particular group of bacteria possessing a gelatinous matrix (Butterfield, 1935; Heukelekian and Littman, 1939). Support in this theory was eroded when numerous other bacterial species, many of which were capable of floc formation, were isolated from sludge (McKinney and Horwood, 1952; McKinney and Weichlein, 1953). Most investigators now agree that *Zooglea* growths are not always involved in floc formation, and these two phenomena may not even be directly related (Pavoni *et al*., 1972).

Sezgin *et al*. (1978) described the filament backbone model at two levels as termed the ‘microstructure’ and the ‘macrostructure’. The microstructure is defined by process of microbial adhesion, aggregation, and bioflocculation. It is the basis for floc formation because, without the ability of one microorganism to stick to another, large aggregates of microorganisms such that exist in activated sludge would never form. The macrostructure of activated sludge is provided by filamentous microorganisms. These organisms forming the backbone of the floc structure provide a skeleton-like structure for flocs and give the floc its strength and integrity. Urbain *et al*. (1993) stated that filamentous organisms were present in all their activated sludge samples taken from different wastewaters and but it was not always associated with poor settling.

DLVO (Derjaguin, Landau, Verwey, and Overbeek) theory, also named as double layer theory, is a classical colloidal theory that describes charged particles as having a double layer of counter ions surrounding the particle. The first layer is often referred to as the Stern layer which is comprised of a tightly associated layer of counterions, and the second layer is often referred to as the diffuse layer which is made up of less tightly associated counterions (Adamson, 1990). The concentration of ions in the diffuse layer decreases with distance from the particle surface until the concentration of ions equals that of the bulk solution. The result is an electric potential that develops around the particle. This double layer or cloud of ions surrounding the particle results in repulsion of adjacent particles and inhibits aggregation. As the ionic strength increases, the size of the double layer decreases, which decreases the repulsion between particles, allowing short-range attractive forces to promote aggregation. The addition of cations to a solution would therefore result in an improvement in bioflocculation due to a decrease in the size of the double layer and the repulsive forces between particles (Sobeck and Higgins, 2002). Zita and Hermansson (1994) investigated the effect of cell surface charge in attachment of bacteria to sludge flocs and observed that ionic strength affects the floc stability. They also stated that these effects could be explained by DLVO theory and K^+ and Ca^{2+} caused similar effects on floc stability.

Azeredo *et al*. (1999) studied the role of exopolymer produced by *Sphingomonas paucimobilis* in the adhesion of these bacteria to glass. The results were interpreted in terms of DLVO and XDLVO (extension of DLVO theory including polar interactions) theories. DLVO was able to explain the results in phosphate saline buffers, although it could not explain the results obtained in the presence of exopolymer. The XDLVO theory enabled the interpretation of the results in the presence of exopolymer, where hydrophobic interactions played an important role. However, polymeric interactions that were not taken into account in these two theories were also expected to be determinant in the adhesion process. Moreover, microelectrophoretic mobility readings were recorded throughout an entire bacterial growth cycle on a culture utilizing glucose as its sole carbon source. The results depicted a relatively constant bacterial surface charge throughout all growth phases

regardless of the flocculability of the culture. Surface charge reduction, therefore, was not considered a necessary precursor to biological flocculation (Pavoni *et al*., 1972).

The polymer bridging model of coagulation developed by LaMer *et al*. (1957), Healy and LaMer (1962), Lamer and Healy (1963). As mentioned previously, electrostatic surface charge reduction to zero for flocculation to occur according to this theory. It was postulated that the adsorption of the coagulant on the dispersed solids surface is first induced at specific chemically active sites where the free ends of polymer segments can come into close contact with points on the uncoagulated colloid or cell. Physical attachment of some of these segments leads to the formation of relatively strong chemical or electro chemical bonds which, because of short distances involved, are likely to overhelm any local electrostatic repulsion between particles having the same net charge (Ganczarczyk, 1983). This classical polymeric flocculation concept subsequently applied to cell aggregation by Tenney and Stumn (1965), Busch and Stumn (1968) and Ries and Mayers (1968) in order to explain the mechanism of floc formation.

Actually the correlation between the accumulation of extracellular polysaccharide material and bacterial flocculation was first observed by McKinney (1953, 1956). He postulated that the envelopment of the cell wall by the polysaccharide material served to displace the attractive forces between similar cells, thereby reducing the effective critical potential on the cell so that aggregation could be achieved. In view of these observations, with polymer bridging model, bioflocculation can be viewed as the result of the interaction of naturally produced, high-molecular-weight, long-chain polyelectrolytes with bacterial cells in such a fashion that the polyelectrolytes bridge the otherwise individual cells into an aggregate that will subside from suspension under quiescent conditions (Pavoni *et al*., 1972).

Furthermore, Busch and Stumn (1968) investigated the effects of both biopolymers and the synthetic polymers on aggregation of microorganisms. They stated that natural polymers isolated from well flocculating bacteria or from ‘slime’producing algae or other plant biocolloids may be more expedient flocculants than the synthetic

polymers since they formed stronger interaction forces. Bioflocs were not as sensitive to dispersion by shear forces as bacterial aggregates flocculated by synthetic polymers.

To be more precise, when there are organic polymers and colloid surfaces as activated sludge flocs, the interaction mechanisms responsible are complex formation by hydrogen bonding, proton transfer and anion interchange with the adsorbed anions. It is widely known that activated sludge polymers contain number of functional groups such as hydroxyl and negatively charged carboxyl groups (Busch and Stumn, 1968; Forster, 1985; Kakii et al., 1989; Higgins and Novak (1997c). The fact that synthetic polymers containing similar anionic (-COO^- and –SO_3^-) and nonionic (- OH) groups are able to aggregate organisms is considered a valid demonstration that natural anionic polymers may, under suitable conditions, become specifically adsorbed at microbial surfaces and thus able to form bridges with adjacent surfaces leading to aggregates. This hypothesis is further reinforced by the observation that interfacially active anionic material precipitated from a cell-free supernatant of a flocculating activated sludge culture can destabilize dispersions of bacteria and of abiotic sols (Busch and Stumn, 1968).

Metal ion bridging is another mechanism for the attachment of anionic functional groups to negatively charged surfaces (Busch and Stumn, 1968). Pavoni *et al*. (1972) investigated the flocculation of kaolinite suspension using either extracted purified polymer or centrifuged bacterial supernatant fluid containing extracellular polymer as flocculating agent. It was observed that at pH 7 only about 25 mg/L exocellular polymer contained in bacterial supernatant was required to achieve optimum removal of a 15 mg kaolinite suspension whereas the optimum dosage of 50 mg/L of extracted purified polymer was required for a 15 mg suspension of kaolinite at the same pH. They concluded that some constituent other than polymeric material in the bacterial supernatant liquid was enhancing its flocculation capabilities and this constituent probably was cation concentration.

The findings of other researchers also confirmed that polyvalent ions aid in flocculation by bridging negative sites on extracellular polymers (Tezuka, 1969;

Steiner *et al.*, 1976; Bruus *et al*., 1992; Dignac *et al*., 1998). Recently, Salehizadeh *et al*. (2000) reported that cations simulate flocculation by neutralization and stabilization of residual negative charges of carboxyl group of uronic acid, pyruvic acid and acetic acid in an acidic polysaccharide forming bridge. High amounts of Ca^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+} were observed to improve dewaterability significantly (Jin *et al*., 2004). It was also shown that Fe^{3+}in solution participated in bridging between the negatively charged groups of EPS and contributed to the improvements in the organic matter in reactor effluent, such as COD, dissolved organic carbon, protein and polysaccharide by decreasing the concentrations of these constituents in the effluent (Li, 2005).

Among the polyvalent metal ions the role of divalent cations (especially Ca^{2+} and Mg^{2+}) have been emphasized by many researchers (Forster and Lewin, 1972; Bruus *et al*., 1992; Murthy *et al*., 1998; Murthy and Novak, 1999; Higgins and Novak, 1997a,b,c; Biggs et al., 2001; Sobeck and Higgins, 2002). In the early research Busch and Stumn (1968) revealed that the role of divalent cations is the formation of complexes or ion pair formation between the functional groups of EPS and the bacteria. Tezuka (1969) also reported that calcium and magnesium were important in floc formation during the growth of monocultures. According to divalent cation bridging theory, divalent cations bridge negatively charged functional groups within the EPS and this bridging helps to aggregate and stabilize the matrix of biopolymer and microbes and therefore promote bioflocculation (Sobeck and Higgins, 2002). A demonstration of the divalent cation bridging model is shown in Figure 2.1.

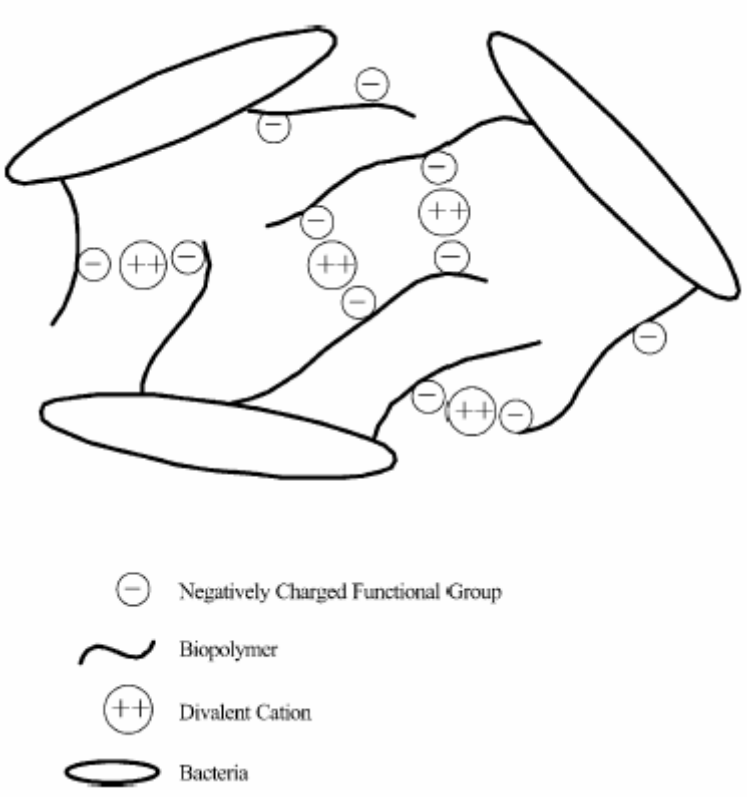


Figure 2.1. Depiction of divalent cation bridging within floc matrix (Sobeck and Higgins, 2002).

Two different mechanisms have been proposed in the uptake of Ca^{2+} and Mg^{2+} by extracellular polymers. The first mechanism is conventional chemisorption by biopolymers at the immediate surface and the second one is more complex and more extensive uptake of metals by the more diffuse polymers. It is suggested that the former interaction occurs with carboxyl groups of polymers whereas the latter occurs with the neutral sugars of the polymers (gels) (Forster and Lewin, 1972).

The studies of Higgins and Novak (1997a,b) supports the divalent cation bridging theory as they observed that the addition of sodium ion caused a deterioration in floc structure affecting the physical properties of sludge due to the displacement of divalent cations (Ca^{2+} and Mg^{2+}) from the binding sites within the floc. Using bactopeptone as feed to the reactors they also observed that the increase in the divalent cations in the feed to the reactors was associated with an increase in bound protein content and concluded that divalent cations act to bind protein and not polysaccharide within the floc (Higgins and Novak, 1997a,b).

When the exocellular protein was characterized it was seen that the protein was a lectin-like protein and binding site inhibition studies demonstrated that the protein had lectin-like activity. The presence of a single protein (lectin) in the exocellular biopolymer fraction suggested that the bound protein fraction is involved in the binding of sugar residues on polysaccharides. Thus, a new model of bioflocculation was proposed. In this model (Figure 2.2.) lectin-like proteins bind polysaccharides that are cross-linked to adjacent proteins and divalent cations also bridge negatively charged sites on exocellular biopolymers (Higgins and Novak, 1997c).

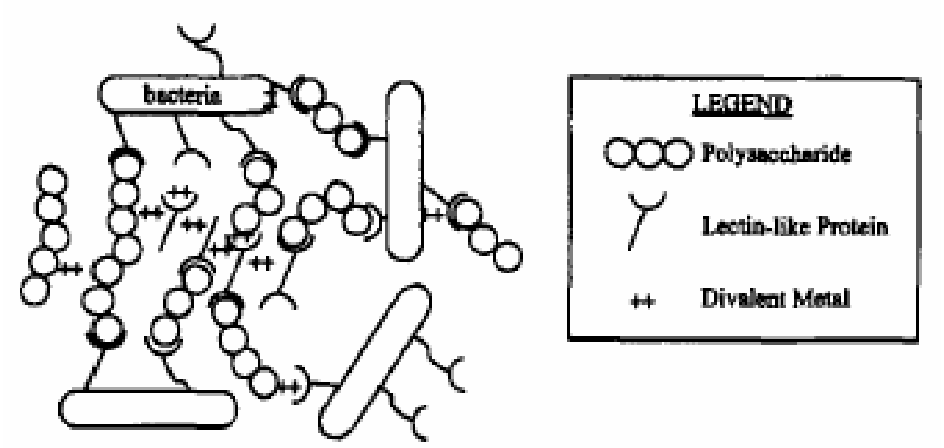


Figure 2.2. Depiction of Role of Lectins in Aggregation of Bacteria by Cross-Linking Polysaccharides and Role of Cations in Binding Biopolymer and in Binding Site Activity of Lectins (Higgins and Novak, 1997c).

On the other hand some researchers proposed that calcium is the most important cation for the ability of bridging between exopolymers themselves and between exopolymers and bacteria (Forster and Lewin, 1972; Kakii *et al*., 1985; Higgins *et al*., 2004). Moreover, studies have shown that removal or replacement of calcium in activated sludge floc weakens the floc structure leading to the appearence of smaller flocs (Eriksson and Alm, 1991; Bruus *et al*., 1992; Sanin and Vesilind, 2000).

According to the calcium ion adsorption results obtained by Steiner *et al*. (1976) there were distinct differences in the behavior of the polymer when it was in the soluble form and when it was either in the solid state or bound to a solid medium. This was attributed in the chemical nature of the adsorption sites and therefore to the strength of the binding. Adsorption by soluble polymers appeared to be by salt formation with carboxyl groups which will, at normal pH values, be practically irreversible. Adsorption by solid polymers can only be explained by postulating an attachment by weak electrostatic forces to a diffuse electron zone originating from one or more of the hydroxyl groups present in the hexose and pentose molecules in the polysaccharide. As the hydroxyl groups would almost certainly be hydrated, adsorption of the metal ion would result in a decrease in the bound water content of the polymer. Such a decrease has been reported by Forster and Lewin (1972).

Keiding and Nielsen (1997) reported that even small changes in the content of calcium in the sludge particles promoted desorption of organic macromolecules and floc disintegration indicating two important things. First, some EPS components were bound by very weak forces, thus being very important for the colloidal stability of the flocs. Second, the cation calcium and not the general ionic strength seemed to play a key role in determining the surface charge of the particles and thus the binding of some macromolecules. Based on these results they suggested a model of typical activated sludge floc (Figure 2.3.) where a ‘cloud’ of organic macromolecules and single bacteria are attached with very weak forces to a more rigid backbone consisting of fibers, filamentous bacteria and bacterial colonies. The association between bacterial colonies and the fibrous structure largely remains intact, whereas most of the EPS and single-cell bacteria are desorbed.

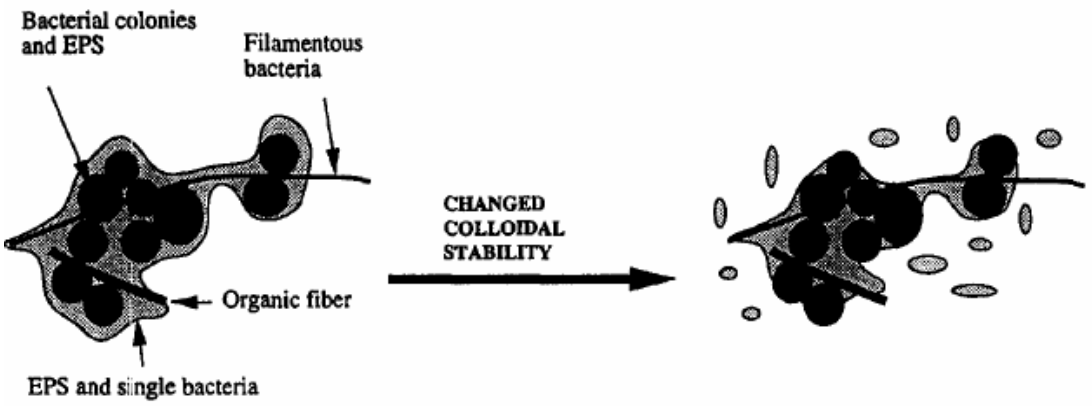


Figure 2.3. Schematic illustration of the changes in activated sludge floc structure, as increased colloidal stability is obtained by changes in the chemical environment of the floc.

One of the suggested mechanisms of floc formation in the literature which is related to Ca^{2+} ions is the formation of an alginate-like gel, which constituted the backbone of the floc structure. (Bruus *et al*.,1992; Sanin and Vesilind, 1996). Alginates are exoplymers consisting of two substantial monomers: 1,4-linked α-L-guluronic acid and β-D-mannuronic acid (Bruus *et al*., 1992). The unique composition of this polysaccharide results in the formation of alginate gels in the presence of calcium ions. This gel is typically referred to as the egg-box model, and is depicted in Figure 2.4. Several bacteria that are known to produce alginate such as *Azotobacter* sp. and *Pseudomonas aeruginosa* (Nunez *et al*., 2000; Davies and Geesey, 1995) have been identified in activated sludge (Pike and Curds, 1971). This suggests that alginate may be present in activated sludge and this mechanism was named as *alginate theory* by Sobeck and Higgins (2002). The alginate theory could be considered a subset of divalent cation bridging theory and polymer bridging theory, but the divalent cation bridging theory suggests a non-specific binding of divalent cations rather than the specific interaction and gel formation between calcium and alginate (Sobeck and Higgins, 2002).

Because of the fact that alginate aggregation is specific for calcium, the investigators inferred that calcium induced aggregation of alginate was important to the

bioflocculation process. Bruus *et al*. (1992) reported that the addition of Mg^{2+} caused a similar exchange of Ca^{2+} within the floc and deterioration in floc properties. As a result, they concluded the biopolymer had greater affinity for Ca^{2+} than Mg^{2+} which supports the role of alginate in bioflocculation (Sobeck and Higgins, 2002).

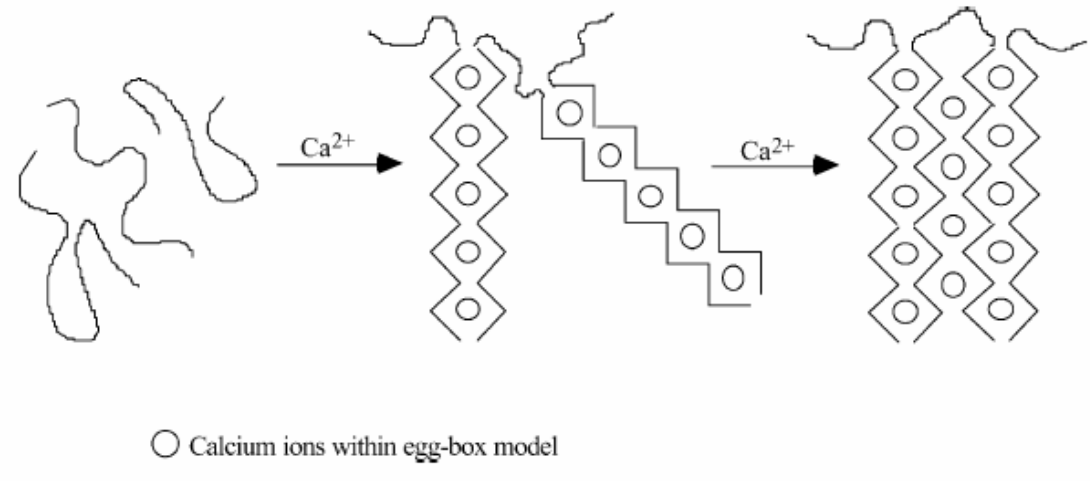


Figure 2.4. Alginate gel formation and egg-box model in the presence of calcium (Sobeck and Higgins, 2002).

In some studies, uronic acids present in polysaccharides have been indicated to promote bioflocculation (Takeda *et al.* (1994), Bender *et al.* (1994), Steiner *et al.* (1976)).There are numerous suggestions that the negatively charged uronic acids in polysaccharides (specifically alginates) interact with divalent cations through charge bridging to promote bioflocculation (Murthy, 1998).

A typical floc consists of a great variety of bacteria and occasionally other organisms such as protozoa, fungi, and viruses, as well as abiotic suspended matter. Thus, a floc may comprise a broad spectrum of hydrophobic and hydrophilic interfaces (Busch and Stumn, 1968). Daffachio *et al*. (1995) also stated that extracellular polymers are known to contain both hydrophobic and hydrophilic groups and it is the net effect of the two components that results in the overall hydrophobicity of sludge.

Hydrophobic interactions may be fundamental in the cohesiveness and other properties of bacterial aggregates called flocs (Jorand *et al*., 1998). Hydrophobic interactions (or 'hydrophobic effect'), result from the behaviour of entities (particles or molecules) incapable of interacting electrostatically or establishing hydrogen bonds with water and are therefore drawn together when plunged in an aqueous phase (Magnusson, 1980). Urbain *et al*. (1993) reported that hydrophobic molecules such as lipids or proteins from the cells can be trapped into the flocs and hydrophobic interactions more specifically internal hydrophobicity of sludge particles are believed to act as essential adhesives between the cells. They have presented a sludge floc model including the hydrophobic interactions together with the cationic bridging of EPS and other polymeric interaction with floc constituents (Figure 2.5.).

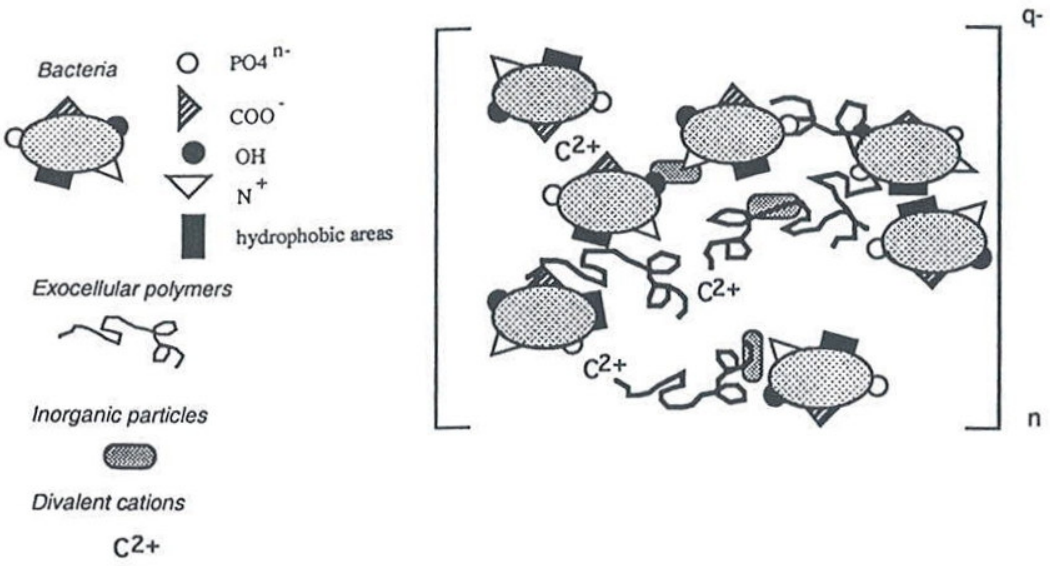


Figure 2.5. Schematic representation of the activated sludge floc (Urbain *et al*., 1993).

Bengtsson (1991) observed that association of bacteria with mineral soil surfaces is determined by contributions from both hydrophobic interactions which are most likely not provided by polysaccharides and specific polymer binding. The polymer produced can be used to overcome the electrical repulsion between the negatively charged surfaces and the bacteria. Zita and Hermansson (1997) indicated that a low

level of cell surface hydrophobicity might be the reason why free-living cells do not attach to flocs. These cells escape sedimentation in the treatment system and reduce the quality of the effluent.

Dignac *et al*. (1998) found that alanine, leucine and glycine were important aminoacids in extracellular proteins. Their hydrophobic properties suggested that they are likely to be involved in hydrophobic bonds. They were nevertheless better extracted by cation exchange resin, providing that electroststic and hydrophobic bonds are closely combined in the flocs, as pointed out by Urbain *et al*. (1993).

Jorand *et al*. (1998) found that a significant proportion of the EPS fraction (at least 7% in terms of dissolved organic carbon) is hydrophobic and the hydrophobic fraction is made up of proteins but not carbohydrates. In addition, 50% of precipitable EPS at pH 2 contained protein and carbohydrate (77% and 66% respecticely), which supports the idea that large quantities of EPS are glycoproteins. Thus they concluded that EPS may be involved in floc cohesion in two ways: (i) through the hydrophilic chains, represented by polysaccharides creating a matrix in which bacteria are embedded and (ii) through a glue creating bridges or reticular points between polysaccharides represented by hydrophobic heteropolymers.

None of the aforementioned mechanisms are alone responsible for the whole floc forming interactions in activated sludge systems. It is probable that combinations of these mechanisms together with other ways of interactions take place simultaneously in flocculation phenomena.

2.5. Properties of Activated Sludge and Their Relationship to Bioflocculation

The literature reveals that extracellular polymers are the key constitutens of bioflocculation mechanism which in turn affects the subsequent behavior of activated sludge systems. The settleability, dewaterability and flow characteristics of sludge as well as its surface chemical properties are all dependent on its flocculated nature. Therefore, understanding of sludge properties is crucial for process efficiency and effluent quality in activated sludge treatment systems.

2.5.1. Rheology

Rheology (from Greek 'rheos' – flow and 'logos' – knowledge) is the science of flow phenomena and related to the viscous characteristics of sludge (Slatter, 1997). Viscosity is classically defined as rate of displacement of a fluid with a given shear force (Vesilind, 1979), thus, rheology describes the deformation (strain) of a body under the influence of stresses (Dentel, 1997).

The design of pumps and pumping system requires knowledge of the rheological properties of the sludge so that friction head losses through pipelines can be calculated (Forster, 1982). The science of rheology as it is known today owes its origin to Sir Isaac Newton who postulated a direct proportionality relationship between the shear stress and shear rate in a fluid (Slatter, 1997). This relationship is given as:

$\tau = \mu (du/dy)$……(2.1)

where τ = shear stress, μ = viscosity, and du/dy = velocity gradient, or shear rate.

A Newtonian viscosity may be considered as a constant for a given fluid or suspension, although it will generally vary if either the temperature or the concentration of particulate material changes. For relatively dilute suspensions, the relationship between concentration and suspension viscosity is quantified by the Einstein viscosity equation (Heimenz, 1986).

$$\eta = \eta_0 \frac{1+\phi/2}{(1-\phi)^2} = \eta_0 (1+2.5\phi) \quad \text{……(2.2)}$$

where η and η_0 = suspension and solvent viscosity, respectively, and ϕ = volume fraction of suspension occupied by particles and/or polymer.

This equation is a key to the relationship between a sludge's viscosity and its

dewaterability. An increase in ϕ and thus η, can be accomplished not only by increased concentration of solids, but also by the incorporation of water into particle structure. Both the biosolids in wastewater sludges, and the polymer used in their conditioning, are capable of incorporating and orienting substantial amounts of water, and this may have significant effects on sludge, centrate, or filtrate viscosity (Dentel, 1997).

The other type of flow property observed in fluids is Non-Newtonian flow behaviour. The shear rate or velocity gradient, du/dy, is not linearly proportional to the shear stress for non-Newtonian fluids such as wastewater sludges. Non-Newtonian fluids are classified into two groups as: time dependent and time independent. Shear stress-shear rate relationship of Newtonian and time independent non-Newtonian fluids are demonstrated in Figure 2.6. Time independent non-Newtonian fluids are also categorized into three groups: Bingham plastic (or plastic), pseudoplastic and dilatant fluids. For Bingham plastic (or plastic) fluids, although the shear stress-shear rate relation is a straight line like Newtonian fluids, the straight line does not pass through the origin. A finite amount of shear stress (yield stress), τ_y, is required to initiate the flow. The relation that describes this behaviour is given as:

$$\tau - \tau_y = \eta\,(du/dy) \qquad (2.3)$$

where η is the coefficient of rigidity or plastic viscosity that represents the slope of the line. The fluid at rest is assumed to have a three dimensional structure of rigidity sufficiently great to resist the finite stress τ_y, which is often called as yield stress. When this stress is exceeded, the structure is broken down and the fluid diplays the linear relationship similar to the Newtonian fluids. Pseudoplastic behavior is seen in flocculated systems in which the aggregates are broken down by increasing shear rates resulting in a reduction of the solvent immobilized by the particles and thus the viscosity. The equation of the flow curve is defined as:

$$\tau = K\,(du/dy)^n \qquad (2.4)$$

where, K is the fluid consistency index, a term analogous to the viscosity term and n is the flow behavior index. The value of n is smaller than 1 for pseudoplastic fluids and its magnitude shows the amount of deviation from Newtonian flow. This behavior also named as shear thinning. Dilatant type of fluids exhibit a rheological behavior which is opposite to that of the pseudoplastic fluids such that the apparent viscosity of dilatant fluids increase with increasing shear rate, a behavior referred to as shear thickening. When these fluids are at rest, it is assumed that they consist of densely packed particles in which voids are small. Liquid is present only to fill these voids. At low shear stresses or shear rates, the liquid lubricates the passage of one particle pass another. Higher shear rates progressively breaks the dense packing of particles, leaving insufficient fluid to enable the particles to flow smoothly pass one another. Therefore the shear stress increases more than proportionately with the shear rate (Metzner, 1956).

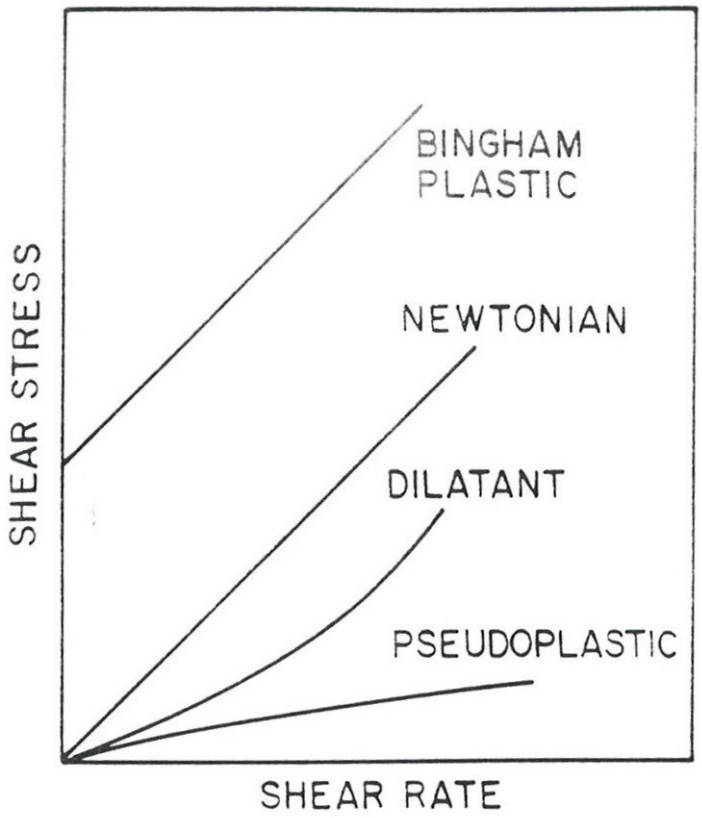


Figure 2.6. Graphical representation of basic fluid flow models

Time dependent non-Newtonian fluids are classified into two groups: thixotropic fluids and rheopectic fluids. For thixotropic fluids the viscosity decreases with the duration of the applied stress. This occurs most probably due to a destruction of floc structures. When the stress is removed, the floc structure reforms, increasing the viscosity (Vesilind, 1979). A thixotropic material is necessarily pseudoplastic but the reverse is not always true (Dick and Ewing, 1967). As to the rheopectic fluids, the shear stress increases with time at a constant rate of shear and it is caused by the similar factors that causes the dilatant behavior.

A very thin activated sludge may exhibit flow properties very close to that of water (Newtonian fluid) whereas, primary or thickened sludge with solids concentration of about 8 to 10% or over is very viscous and have complex flow behavior. Conflicting results have been proposed in classifying the wastewater sludge as Bingham plastic (true plastic) or pseudoplastic (power law) material. Some researchers identified sewage sludge as a pseudoplastic material (Behn, 1962) whereas some others defined it as a Bingham plastic material (Dick and Ewing, 1967; Rimkus and Heil, 1975).

Due to the non-Newtonian behavior of sludge; instead of viscosity, the term apparent viscosity has been used in the literature. Apparent viscosity is the instantenous viscosity which is defined as the ratio of shear stress to shear rate at a fixed shear rate applied, at a fixed time.

The contribution to non-Newtonian behaviour of sludge is believed to originate from the colloidal properties of solids more than from the molecular properties of the suspension (Hiemenz and Rajagopalan, 1997). Considering that the colloidal properties of sludge depend on factors like pH, conductivity, solids concentration and flocculation properties, the effects of these variables on the rheology of activated sludge were examined (Sanin, 2002). Results showed that the solids concentration has the most significant effect on the rheological property of sludge. With increasing solids concentration, pseudoplasic viscosity increases sharply and the flow behaviour index decreases significantly indicating that the flow tends to be more pronounced non-Newtonian at high solids contents. This is an important concern in the design of transport systems for thick sludges. Lowest viscosity was observed at the lowest pH

studied (5.5) which is the value closest to the isoelectric point of bacteria from among the pH values studied. With increasing pH values, the viscosities were observed to increase. Effect of pH was significantly intensified when the solids concentration was increased. Apparent viscosity was found to decrease with increasing conductivity of sludge. This was believed to be due to the compression of the electrical double layer around the particles that results in a more compact floc structure and coiled polymer confomation in and out of the flocs. Presence of extracellular polymers affected the viscosity of sludge as it was also suggested in earlier researches. It was observed that apparent equilibrium viscosity of activated sludge is reduced by the action of cellulase, indicating that the surface polysaccharides have a significant role in determinig the rheological characteristics of activated sludge (Forster, 1982; Forster, 1983). Removal of polymers by multiple centrifugation runs correlated well with the decrease in viscosity (Sanin, 2002).

An examination of the rheological properties of activated sludges from three sewage treatment works and a digested sludge from a dual mesophilic-thermophilic anaerobic digester (TAD-MAD) showed that all the sludges exhibited yield stresses the magnitude of which varied with the suspended solids' concentration and the type of treatment which they had received. The surface charge carried by the sludge particles and the bound water of the sludge were both found to be related to the yield stress (Forster, 2002). It was also reported that metal ions causes a decrease in bound water content and thus the sludge viscosity (Forster, 1983).

2.5.2. Dewaterability

Dewaterability is an important sludge property as it determines how easily the water content of sludge can be reduced in order to decrease the sludge volume. Sludge volume reduction is of crucial significance to facilitate transport and handling, or to minimize the space or energy needed in case of drying or incinerartion (Jin *et al*., 2004).

Water content of sludge is divided into four categories as free water, interstitial water, vicinal waste and water of hydration (Vesilind, 1994). Free water is

not attached to sludge solids and can be separated by simple gravitational settling. Interstitial water is trapped within the floc structure, or perhaps within a cell. This water can be released when the floc is broken up or the cell is destroyed. Some interstitial water might be removed by mechanical dewatering devices such as centrifuges. Water of hydration is chemically bound to the particle and can be released only by thermochemical destruction of particles (Vesilind, 1994).

Many factors that influence the dewatering characteristics of sludge have been reported in the literature. Among these are cellulose content, pH and particle charge, organic content, bound water content, filtrate viscosity, alkalinity, solids concentration, nitrogen content, grease content, conditioning, type of sludge, compressibility coefficient, mechanical strength of particles, porosity, mixing, biological degradation, and particle size. Of all the factors that influence sludge dewatering characteristics, particle size distribution seems to be the property of sludge that most affects its dewaterability. (Karr and Keinath, 1978).

Dewaterability of sludge can be analyzed by either Specific Resistance to Filtration (SRF) or Capillary Suction Time (CST) tests. Specific Resistance to Filtration (SRF) test has been the most commonly applied method to measure dewaterability and used in this study.

Specific Resistance to Filtration test is based on Darcy's equation describing the flow of fluid through porous media (Vesilind, 1979). The test is performed by filtering a known volume of sample under vacuum and volume of filtrate withdrawn from sludge is measured as a function of time. Specific resistance to filtration (r) is then calculated from the slope of the t/V versus V graph using the following formula:

$$\frac{t}{V} = \frac{\mu r w}{2PA^2} V + \frac{\mu R_f}{PA} \quad \text{.....(2.5)}$$

where;

t = Time, s

V = Volume of filtrate, m^3

μ = Viscosity of filtrate, $N.s/m^2$

w = Weight of dry cake solids per unit volume of filtrate, kg/m^3

P = Pressure difference, N/m^2

A = Filtration area, m^2

R_f = Resistance of filter medium, 1/m

r = Specific resistance to filtration, m/kg

Further details of this method are described in section 3.5.2.

Specific resistance to filtration is a property of filtering sludge cake, and can be defined as the resistance to filtration of a unit weight of sludge cake per unit area at a given pressure. Hence, high SRF values indicate poor dewaterability of sludge. The SRF values of activated sludge are reported to be between 10^{10} and 10^{14} m/kg. Other values reported in the literature vary between $1.79*10^{12}$ and $6*10^{13}$ (Pitman, 1975; Karr and Keinath, 1984; Gulas *et al*., 1979; Bowen and Keinath, 1984). Type of sludge whether it is raw, activated or digested affects the value of SRF. Aeration basin condition (Wu *et al*., 1982; Novak, 1986), particle size distribution and conditioning with neutral and synthetic conditioners (Karr and Keinath, 1978; Bruus *et al*., 1992: Eriksson and Alm, 1993) are the other factors affecting the specific resistance to filtration. Lee and Liu (2001) measured the SRF of raw sludge as $1.1*10^{13}$ m/kg.

Filtering characteristics of activated sludge measured are closely associated with the operation of activated sludge system such as the ratio of carbon (as COD) to nitrogen in the wastewater. As the activated sludge waste treatment system is operated at the identical F/M ratio or SRT, the specific resistance of nitrogen-rich activated sludge is consistently lower than that of the nitrogen-deficient activated sludge. Generally the filtering properties of activated sludge are poor owing to the excessive growth of long-length attached or long-length free-floating filamentous microorganisms and the existence of dispersed and pinpoint flocs (Wu *et al*., 1982). Durmaz and Sanin (2003) reported that increase in C/N ratio from 9 to 21 and further to 43, increased

the total polymer quantity favoring the production of higher amount of carbohydrates causing the sludge much harder to dewater.

Surface lipids, proteins, and carbohydrates are all related to the dewaterability and dose of cationic polymers required for conditioning. Proteins and carbohydrates provided attachment sites for the synthetic polymer and decreased the required polymer dose when they are high in surfaces (Bowen and Keinath, 1984). Moreover, experimental results indicate that sludge conditioned with dual (cationic and non-ionic) polymers shows a better dewaterability with less chance of overdosing compared with sludge conditions with a single polymer (Lee and Liu, 2001). Addition of sludge conditioner in excess of the optimum could cause a charge reversal that resulted in a restabilization of the sludge flocs/particles and an increase in specific resistance (Wu *et al*., 1982).

Gulas *et al*., (1979) and Mikkelsen and Keiding (2002) observed that specific resistance of the sludges were inversely related to the biological polymer content on active basis. Eriksson (1987) observed that (using electron microscopy and sludge conditioning) well flocculated sludges contain large amounts of biopolymers. Sanin and Vesilind (1994) reported that, removal of EPS even partly, causes the release of small particles capable of clogging the filtration channels in the filter paper and sludge cake. In addition, High amounts of individual and total polymers in the extracted EPS corresponded to a good dewaterability determined by the CST (Jin. *et al*., 2004).

However, it has been widely reported that large amounts of dissolved and easily extractable polymers indicate badly flocculated flocs making the sludge more difficult to dewater. An increase in floc strength and filterability correlates to a decrease in the amounts of EPS based on the maesurements of SRF (Shiyoma and Toriyama, 1985; Durmaz and Sanin, 2003), and CST (Ericsson and Eriksson, 1988; Eriksson and Alm, 1991; Pere *et al*., 1993). Moreover, Wu *et al*., (1982) reported that the overproduction of extracellular biopolymers could produce a considerably higher surface charge around the sludge causing poor dewaterability.

Novak *et al*. (2003) suggested that the specific resistance to filtration and polymer conditioning requirements increased during both anaerobic and aerobic digestion and these were accompanied by an increase in soluble proteins and polysaccharides. The protein concentration in solution was substantially greater than the polysaccharide under anaerobic conditions; polysaccharides were the major biopolymer remaining in solution under aerobic conditions.

On the other hand, Houghton *et al*. (2001) reported that filterability of activate sludge was source dependent. For the thickened and anaerobic digested sludges, decreasing of EPS resulted the dewareability to improve, but for the activated sludge, dewareablity increased with the EPS content until reaching the maximum level of 35 mg EPS/gSS. Above this value, the dewaterability decreased.

Cation concentration is another parameter which increases dewaterability of sludge as these cations contribute to the floc formation by binding polymeric chains in the flocs together and by decreasing the net negative floc surface charge (Higgins and Novak, 1997b; Keiding and Nielsen, 1997; Wilen *et al*., 2003a). Jin *et al*., 2004 indicated that the bound water content and Capillary Suction Time (CST) were significantly influenced by the concentration of the divalent and trivalent cations (Ca^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+}) such that high concentration of these multivalent cations was related to low values of bound water contents and CST. Furthermore, in a study performed by Bruus *et al*. (1992) the extraction of Ca^{2+} led to an increase in the number of small particles and subsequently an increase in the specific resistance to filtration and the turbidity correlated well with dewaterability measured as specific resistance to filtration.

The tests conducted at the treatment plant revealed that the complex interactions between cations and sludge influenced the settling and dewatering properties in a manner that depended on ratios and concentrations of monovalent to divalent cations in the activated sludge feed and solution (Murthy *et al*., 1998; Higgins and Novak, 1997a; Higgins *et al*., 2004). A ratio of sodium (Na^{+}) to divalent cations greater than approximately two resulted in a deterioration in dewatering characteristics (Higgins and Novak, 1997a). Calcium to magnesium ratios near one were considered optimum

(Higgins and Novak, 1997b). On the other hand, high potassium (K^+) sludges displayed higher floc strength characteristics while sodium sludges did not. The interaction of potassium (unlike sodium) with activated sludge flocs is not completely explained by simple charge competition (M/D ratio) in the divalent charge bridging model. In general, excess sodium (as reported by Higgins and Novak (1997a) always produced poorly settling sludges, poor dewatering and weak flocs. Excess potassium produced poor dewatering but flocs were resistant to shear and settled well (Murthy and Novak, 1998).

2.5.3. Settleability

The efficiency of most activated sludge systems depends on the separation of the solids, and this operation is controlled by the settling characteristics of the mixed liquor suspended solids (Barahona and Eckenfelder, 1984). Settling tests are conducted in the laboratory for the design of the thickeners. The two most common tests performed in the laboratory are zone settling velocity (ZSV) and sludge volume index.

Zone settling velocity is conducted in a transparent one liter cylinder filled with sludge and mixed to distribute solids evenly. It measures the velocity of the interface between sludge solids and clear supernatant as the sludge settles. Zone settling velocity is a strong function of solids concentration and is inversely proportional to it (Vesilind, 1979). ZSV is a key parameter to evaluate the sludge settling properties, since it can be used to determine how much the secondary settlers can be loaded (Jin *et al*., 2003).

Operational characteristics of the aeration basin such as sludge age (Bisogni and Lawrence; Chao and Keinath, 1979), food to microorganisms ratio (Chao and Keinath, 1979) and organic loading (Barahona and Eckenfelder, 1984) affect the type of microorganisms and also characteristics of flocs formed. Barahona and Eckenfelder (1984) suggested that, in the range of organic loading tested (0.1-0.6 g BOD/g MLVSS-day), the ZSV increased with the organic loading due to the size of the sludge floc which was also directly proportional to the organic loading. The

settling characteristics of activated sludge in relation to organic loading were also previously investigated and it was stated that increasing the food to microorganisms (F/M) ratio over certain values caused poorer settling and compaction characteristics of the sludge (Bisogni and Lawrence, 1971; Chao and Keinath, 1979). The ZSV in aerobically digested sludge was found to decrease with sludge age. The addition of commercial polymers did not substantially improve the ZSV (Barahona and Eckenfelder, 1984). However, addition of a fine, mineral, talc-based powder into the aeration tanks of pulp and paper wastewater treatment plant resulted an increase in floc aggregation and the production of heavier and well-structured flocs. The sludge settling velocity is increased and an efficient solid/liquid separation was obtained (Clauss *et al*., 1999).

Sezgin *et al*. (1978) stated that the relative number of filamentous and flocculant microorganisms was one of the most important factors determining the physical characteristics of the floc related to settling properties. Under certain conditions of the aeration tank, filamentous microorganisms overpopulate the other microorganisims and form a lattice structure in the floc. Then they extend out of the floc creating a very high frictional resistance that reduces the sludge settling velocity. This type of sludge having settling problems is termed as 'bulking sludge' which causes sigificant seperation problems in wastewater treatment plants.

Sludge volume index (SVI) is another test to measure sludge settleability. The SVI is defined as the volume in mL occupied by a gram of sludge after 30 min of settling. The critical value of this indice tend to be taken by the authors is 150. If this value is exceeded the sludge is said to have bulked, which in practical terms means that the sludge blanket will have moved towards the top of the settlement tank and there will be a risk of solids being carried over the weir with the final effluent (Goodwin and Forster, 1985). SVI is an operational test performed to designate sludge settleability in a very short time. It does not measure settling velocity but provides a quick and rough data as to whether the sludge is good settling sludge or not (Dick and Vesilind, 1969).

The influence of EPS on sludge settling has been widely studied, but since there is no unified method for their extraction, it is always difficult to compare results from different studies. Thus, relationships between sludge settling and EPS (Magara *et al*., 1976; Chao and Keinath, 1979) are sometimes contradictory (Urbain *et al*., 1993).

Many studies revealed that sludge settled better with lower SVI and that SVI increased with the EPS in sludge (Magara *et al*., 1976; Eriksson and Alm, 1991; Urbain *et al*., 1993; Jorand *et al*., 1994). Moreover, some studies demonstrated SVI decreased with increasing EPS (Goodwin and Forster, 1985) while the others found no correlation between the two properties (Chao and Keinath, 1979; Jorand *et al*., 1998; Liao *et al*., 2001).

Although differences in the quantites of EPS constituents were noted, there is increasing evidence that the composition and properties (e.g. hydrophobicity and surface charge) of EPS is more important with respect to settleability than the amount of EPS produced (Andreadakis, 1993; Bura *et al*., 1998).

Sponza, (2004) showed that decreases in bound protein ratio of total protein in EPS are associated with high SVI values. Similarly, Higgins and Novak (1997a) have associated the increase in bound protein content with improvements in settleability However, Martinez *et al*. (2000) found that SVI increased with protein content of EPS, but did not observe any correlation with carbohydrate or lipid content of EPS. In addition, Novak and Haugan (1981) observed that SVI was also associated with free soluble exocellular polymer. Likewise, Sudhir (1998) showed that excess soluble protein resulted in weak flocs and poorly settling sludges.

Moreover, an examination of the EPS composition, in relation to settlement behavior, has shown that the lipid fraction has significant effect. The general trend is for the lipid concentration to increase as sludge settlement deteriorates (Goodwin and Forster, 1985). However, Bura *et al*. (1998) found that the DNA content was the only constituent identified which had any correlation to settling characteristics (SVI) of the sludge. The studies on municipal sludge revealed that increase in the concentration of exopolymeric carbohydrates decreases the SVI value, whereas

increase in the lipids concentration of EPS increases SVI deteriorating sludge stability (Goodwin and Forster, 1985; Urbain *et al*., 1993.)

The effects of various airflow rates on carbohydrate to protein ratio and sludge settling characteristics were investigated using three modified sequencing batch reactors. No relationship was observed between sludge settling and the amount of EPS. This result contradicted previous research which found that biopolymers played a central role in absorbing and bridging cells and initiating floc formation. This was because the properties of EPS were changed during growth at elevated oxygen concentrations. Higher airflow rate increased the amount of carbohydrate in the EPS and this caused poor settling characteristics (Shin *et al*., 2001).

As C/N ratio of the feed increased, the carbohydrates in the EPS extracts increased sharply and this resulted in the decrease in zone settling velocity and increase in SVI (Durmaz and Sanin, 2003). On the other hand, Andreadakis (1993) could not observe a correlation between carbohydrate content of sludge and sludge settleability. Sludge settleability in systems with a low SRT (2.5 days) is significantly influenced by temperature, with an increased SVI at higher temperature (Krishna and van Loosdrecht, 1999).

Since the composition of EPS was influenced by the substrate type and by the microorganisms in activated sludge, settling characteristics are thought to be strongly influenced by the reactor microenvironment including biomass population dynamics and degradable organics. The flocs grown in wastewater containing readily degradable organics exhibited good settling properties in low SVI values. The flocs grown in wastewater containing organic substrates degradable with difficulty such as chemicals and dyes exhibited poor settling properties (Sponza, 2004)

The metal binding capacitiy of extracellular polymers is another property that affects the settling characteristics of sludge (Tezuka, 1969; Bruus *et al*., 1992; Higgins and Novak, 1997a,b,c; Murthy and Novak, 1998; Sobeck and Higgins, 2002; Jin *et al*., 2003). In contradiction with the DLVO theory, Higgins and Novak (1997a) showed that excessive addition of a monovalent cation, sodium, did not improve even

deteriorated the settling and dewatering properties. A ratio of sodium to divalent cations greater than two caused deteriorations in settling and dewatering properties (Higgins *et al*. 1997a) and the calcium to magnesium ratio near one was considered optimum (Higgins *et al*. 1997c). In another study, for concentrations of less than 8 meq/L of calcium no significant increase in floc size was observed even though an increase in the initial rate of change of floc size was seen. Addition of calcium greater than 8 meq/L resulted in a dramatic increase in floc size and settleability (Biggs *et al*., 2001).

2.5.3.1. Problems in Settleability: Sludge Bulking

The successful application of the activated sludge process requires that the sludge floc settles and compacts well in the settling tank. It must settle well so that the effluent has little suspended solids. High suspended solids in the effluent are the main cause of unsatisfactory effluent quality, and they also make proper SRT control difficult. The sludge must compact well so that it can be returned successfully to the aeration basin. A highly compacted sludge also reduces the costs for dewatering and disposal of the wasted solids. The most pervasive and difficult problem among these solids separation problems is the *sludge bulking* which is defined as the formation of activated sludge *floc that settles slowly and compacts poorly* (Rittman and McCarty, 2001).

The onset of sludge bulking can be observed in three ways. First and the most direct way is microscopic examination. Regular microscopic examination by a trained technician can identify when extended filaments are increasing. A steady trend of more extended filaments is a sign that bulking is at hand. Second, the sludge volume index is well correlated to bulking. An SVI greater than about 200 mL/g usually indicates serious bulking. Very bad bulking can have SVIs much greater, perhaps as much as 500 mL/g. The third sign is the combination of a rising sludge blanket and a low concentration of suspended solids in the settler underflow (Rittman and McCarty, 2001).

Two types of sludge bulking mechanisms have been suggested in the literature; the first one is *filamentous* bulking and the second one is *non-filamentous* bulking which are described as follows.

2.5.3.1.1. Filamentous Bulking

In this type of bulking, filamentous organisms grow in profusion. They grow both inside the flocs and outside the flocs, penetrating into the bulk solution. They can stretch out the floc, making it diffuse, and/or bridge in between flocs, thereby interfering with their close approach, just like mechanical protrusions. Such a sludge settles and compacts poorly and it will have a high SVI (>150 mL/g). When such sludge settles it produces an extremely clear supernatant because the large numbers of extended filamentous organisms filter out the small particles that cause turbidity (Jenkins *et al*., 1993).

Poor sludge settling, as expressed by SVI, is observed when the length of extended filaments exceeds 10^7 μm/mg suspended solids. More than 30 types of filamentous microorganisms have been identified in bulking activated sludge (Eikelboom, 1975; Palm *et al*., 1980; Sezgin, 1982; Sezgin *et al*. 1978, 1980; Bitton, 1994). *Microthrix parvicella*, Eikelboom types 0041, 021N, 0092, 0675, 1863, *Thiothrix*, *Nocardia spp., Haliscomenobacter hydrossis* and *Sphaerotilus natans* have been identified as the most common filamentous organisms involved in bulking (Madoni *et al*., 2000; Ramothokang *et al*., 2003).

The settling, compaction and separation properties of an activated sludge are related to the relative numbers of filaments and floc-forming organisms. The outgrowth of excessive quantities of filaments from the floc is correlated with sludge-bulking, whereas the absence of filamentous organisms leads to the production of small, weak flocs which settle poorly (pin-point flocs). An ideal sludge is found when there is a balance between filamentous and floc-forming organisms (Horan, 1990).

There are major physiological differences between floc-forming and filamentous bacteria. Filamentous bacteria have a higher surface-to-volume ratio than their floc-

forming counterparts, and this helps them to survive under low oxygen concentrations and low-nutrient conditions. They also have a low half-saturation constant and have a high affinity for substrates, thus behaving as oligotrophs and surviving well under starvation conditions. Filamentous bacteria are able to predominate under low dissolved oxygen, low F/M, low-nutrient conditions, and high sulfide levels. However it appears that low F/M is the predominant cause of bulking in wastewater treatment plants (Bitton, 1994).

It was reported that excessive growth of filamentous microorganisms does not cause a bulking situation in all cases (Pipes, 1979; Wanner, 1992). The volume fraction of extended filamentous bacteria in the activated sludge culture that causes settling problems could be minor (Martins *et al*., 2004). According to Palm *et al*. (1980) and Kappeler and Gujer (2002) volume fractions of 1-20 % are sufficient to cause bulking sludge. Kaewpipat and Grady (2002) even suggested that the number of filamentous bacteria in bulking sludge can be too low to detect by denaturing gradient gel electrophoresis (DGGE). This would indicate that the filamentous bacteria regularly do not represent the dominant metabolic bacterial group in the treatment plant, but still cause bulking sludge (Martins *et al*., 2004). Futhermore, some authors also stated that the presence of filamentous bacteria was not always associated with poor settleability (Urbain *et al*., 1993; Thompson and Forster, 2003).

The research performed by Thompson and Forster (2003) revealed that addition of magnesium ions was successful in eliminating filamentous bulking conditions and addition of calcium to that feed brought about an appreciable improvement in settlement characteristics of sludge from paper mill wastewaters.

2.5.3.1.2. Non-filamentous (Viscous) Bulking

The sludge bulking condition occurred in the absence of filamentous microorganisms is named as ‘zoogleal bulking’ (Eikelboom and van Buijsen ,1981) and/or viscous bulking (Hale and Garver 1983; Jenkins *et al.*,1993) depending on the viewpoints of the researchers.

In the previous studies the reason of non-filamentous bulking was attributed to the excessive growth of *Zooglea*-like microorganisms (Eikelboom and van Buijsen, 1981).Generally, zoogleal microorganisms form two types of colonies: fingered and amorphous. If the zoogleal microorganisms are present in large amounts, the in exocellular slime causes the sludge flocs to have a voluminous character resulting poor settling and compaction. However, it was later revealed that not only the zoogleal microorganisms but also other microorganisms producing slime substances in excess can cause similar irregularities (Novak *et al*., 1993).

The later approach states that non-filamentous (viscous) bulking is caused by microstructure failure in which too much of the extracellular material that contributes to bioflocculation is produced (Jenkins *et al.*, 1993). Microbial cells are 'dispersed' in an extracellular mass which can become highly water retentive as the biopolymers are hydrophilic colloids (Wanner, 1994). Since this condition can result in a viscous, poorly-settling and compacting activated sludge, it is possible that it constitutes one of the conditions that previously has been referred to as non-filamentous bulking (Pipes, 1979). This condition can be visualized microscopically by reverse staining with India ink (Jenkins *et al.*, 1993).

Activated sludge from domestic wastewater treatment plants normally contains 14% to 18% total carbohydrates (expressed as glucose on a dry weight basis by the anthrone test). Industrial wastewater treatment activated sludges may contain up to 20% to 22% carbohydrate expressed as glucose on a dry weight basis. In severe cases of 'slimy sludge' or 'viscous bulking,' carbohydrate levels of up to 70% as glucose on a sludge dry weight basis have been seen. Interference in sludge settling starts to occur at an activated sludge carbohydrate content of approximately 25% to 30% as glucose (Jenkins *et al.*, 1993).

Overproduction of extracellular biopolymers can also lead to other kinds of separation problems. Polymers make the activated sludge sticky, and when the thickened layer of sludge is not periodically mechanically disturbed, the activated sludge loose its ability to flow. This phenomenon was observed in activated sludges from phosphorus removal systems. The biocenoses of the sludges were always

dominated by large grape-like clusters of polyphosphate-accumulating bacteria (Neisser positive), and the activated sludge settled quite well. Such a decreased ability to flow can cause trouble with removing the thickened sludge layer from secondary settling tanks (Wanner, 1994).

2.5.3.2. Reasons of Bulking

The factors influencing filamentous organism growth in activated sludge can be classified into 'general factors' and 'specific factors'. The general factors are; sludge residence time or food to microorganisms ratio, aeration basin configuration (or wastewater feed regime), presence of initial unaerated zones (anoxic or anaerobic) in the aeration basin where the return activated sludge and the influent wastewater mix together. Specific factors influencing the occurrence of filamentous organisms in activated sludge are; dissolved oxygen concentration, nutrient (N and P) concentration, pH, nature of organic substrate including whether it is soluble or particulate and whether it is readily biodegradable or slowly biodegradable, seeding from surfaces, surface trapping of foam and foam recycle (Jenkins *et al*.,1993).

Pernelle *et al.* (2001) studied the influences of different applied stresses such as; oxygen shortage, transient substrate overload with added oxygen and transient substrate overload coupled with oxygen storage on filamentous organisms. There seemed to exist a relation between a kind of stress and a filamentous microorganism belonging to a specific group rather than between a kind of stress and a specific microorganism. Only *H. Hydrossis* was greatly favoured by all of these three conditions. This work has shown that a massive input of easily assimilable substrate or an oxygen deficiency alone, are not enough to induce bulking whereas a combination of the two facilities is.

Viscous bulking usually occurs when wastewaters high in readily metabolizable, soluble organics are treated under nutrient (N and/or P) deficient conditions. Apparently the exocellular slimes are products of a 'shunt metabolism' or unbalanced growth, formed by the activated sludge microorganisms (both floc formers and filaments) when they cannot produce nitrogen- and phosphorus-

containing cell material due to lack of nutrients (Jenkins *et al.*, 1993). Among the above factors, nutrient deficiency is of prime importance in the context of our study.

In bacterial growth, elements like nitrogen, phosphorus and sulphur are termed macronutrients because of their significant content in microbial biomass. In addition, the elements such as Fe, Ca, Mg, K, Mo, Zn and Co can be classified as micronutrients. The mass fraction of these elements in biomass is negligible, but they play an important role (Wanner, 1994).

Nutrient deficiency in wastewater or during the treatment process can have adverse effects on the process such as incomplete conversion of organic and inorganic substances to end products. Inefficient removals of BOD_5 or COD often have been reported as resulting from deficiencies in nitrogen and phosphorus. Inhibition of nitrification because of iron deficiency also has been reported (Gerardi, 1994).

Nutrient deficiency also can result in a shift in the microbial population of the process. Filamentous organisms can effectively use some nutrients that are in low concentrations. Consequently, they grow faster than non-filamentous organisms under nutrient deficient conditions as also mentioned previously. A change of microbial population to filamentous bacteria, fungi, and actinomycetes (*Nocardia* and related organisms) frequently has been observed in activated sludge processes as a result of nutrient deficiency (Gerardi, 1994).

Diagnosis of nutrient deficiency can be by a combination of wastewater analysis and microscopic examination of the activated sludge. If a microscopic examination of the activated sludge reveals the presence of any of the following, then nutrient deficiency is to be suspected:

- Major filament types: 021N or *Thiothrix* spp.;
- Viscous activated sludge showing significant amounts of extracellular matrial by India ink reverse staining or detection of a polysaccharide content of greater than 20 % to 25% on a dry weight basis using the anthrone test;

- Foam (scum) on activated sludge aeration basins and secondary clarifiers containing significant amounts of extracellular material (often Neisser positive) and not containing *Nocardia* spp., *M. parvicella* or type 1863.

Poor activated sludge settling due to viscous bulking caused by nutrient deficiency cannot be satisfactorily controlled by chlorination or H_2O_2 addition. It can be made to settle with difficulty by large doses of polymer. There is danger that, should the nutrient deficiency become more severe, the ability to remove soluble organic matter will be lost and the treatment will fail.

In cases where the microscopic examination suggests nutrient deficiency, the BOD_5, COD, or TOC to N and P ratios of the wastewater influent to the aeration basin and the total P and TKN content of the mixed liquor suspended solids (MLSS) should be analyzed and if necessary, the required nutrient(s) should be added. It has often been stated that sufficient nutrients are present when the wastewater to be treated by activated sludge contains BOD_5/N/P in the weight ratio 100/5/1 (Jenkins *et al.*,1993).

With sufficient phosphorus source, the effects of nitrogen deficiency on activated sludge bulking were studied in an SBR fed with brewing process wastewater. The experimental results showed that the sludge settled properly at an influent BOD/N value of 100/4. When the value of BOD/N was 100/3, filaments had an excessive growth at one time during the reaction process. Afterwards, the number of filamentous bacteria began to decrease and simultaneously an excessive growth of viscous zoogloea with high percentage of moisture was observed and viscous bulking occurred. When the influent BOD/N value was 100/2, the excessive growth of filamentous microorganisms was not observed at all and the sludge characterization was similar to the case in which BOD/N value was 100/3. When the value of influent BOD/N was 100/0.94, a more serious viscous bulking occurred (Peng *et al.,* 2003)

Ericsson and Eriksson (1988) studied the influence of low soluble phosphorus concentrations on the activated sludge for optimization of chemical pre-precipitation in Enköping. During extreme pre-precipitation conditions, the sedimentation characteristics of the activated sludge deteriorated with a very marked increase of

sludge volume index and growth of filamentous organisms. Specialized laboratory studies showed that wide variations of pre-precipitation dosages used in the plant trial operation apparently caused shifts in microbial population. This was indicated by variations in the amount of filamentous bacteria. They concluded that easily assimilated phosphorus depletion favors filamentous organisms' growth.

On the contrary, the other important observation was that the nutrient deficient reactors did not contain mainly filamentous bacteria. Microbiological analysis results showed that the survival of *Nocardia sp*. which is a filamentous type of bacteria was observed after long term operation under poor phosphorus (P deficiency) conditions in dye industry flocs (Sponza, 2002).

Furthermore, Wanner (1994) stated that holding cells for a certain time under phosphorus starvation followed by exposure to phosphorus can lead to a great accumulation of polyphosphate, e.g., the so-called polyphosphate over-plus, which may account for much of the 'luxury uptake' observed at times in the activated sludge process. As McGrath *et al*. (2001) defined, polyphosphate (poly-P) is a linear polymer of phosphate residues linked together by high-energy phosphoanhydride bonds and may account for up to 10-20 % of the cellular dry weight (Kornberg *et al*., 1999; Kulaev, 1979). The primary purpose of the stored polyphosphate in most bacteria is a phosphorus source for periods of phosphorus starvation (Wanner, 1994).

Khoshmanesh et al. (2002) also reported that under aerobic conditions when only acetate was available, poly-P accumulating organisms took up phosphorus as polyphosphate granules inside the cell. In fact, some cells poly-P granules occupied almost the total cell volume. The rate of phosphorus ingestion under aerobic condition appears to be dependent on the unsaturated storage capacity for the intracellular phosphorus as well as upon the concentration of extracellular phosphorus (Somiya *et al*., 1986).

McGrath *et al*. (2001) designated that intracellular polyP has previously been shown to play an important role in the physiological adaptation of microbial cells during growth and development, and in their response to nutritional and environmental stress (Kornberg, 1995). For example, extensive accumulation of polyP in

Escherichia coli was reported in response to osmotic stress or to nutritional stress imposed by nitrogen, amino acid or phosphate limitation (Ault-Riche *et al*., 1998).

The polyphosphates in the cells together with lipidic and proteinaceous materials forms intracellular granules called *volutine* after *Spirillum volutans*, (Wanner, 1994) a bacterium in which these granules were observed for the first time (Schlegel, 1985). Incorporation of metal ions in the structure of volutin granules was also reported by many researchers as a result of X-ray analysis and appear as spherical, dark, electron-dense bodies under electron microscope. (Buchan, 1981; Röske *et al*., 1989; Bonting *et al*., 1993; Schönborn *et al*., 2001). Similarly Bura *et al*. (1998) detected electron dense particles, identified by energy dispersive spectroscopy as containing iron, phosphorus and sulfur in the matrix of the floc grown under P-depleted and P-limited conditions when observed under electron microscopy.

Because of the fact that phosphorus occurs in aquatic systems mainly as the negatively charged species orthophosphate (PO_4^{3-}), cations were found to be associated with to satisfy electroneutrality. Potassium and magnesium, and to a lesser extent, calcium were the cations reported to be associated with phosphorus for this purpose (Patterkine, 1991 and 1999).

Machnicka *et. al*. (2004) stated that phosphorus accumulation by floc-forming bacteria was well known and often confirmed. Hence, they compared the phosphorus uptake by filamentous microorganisms present in the foam and non-filamentous activated sludge bacteria and examined the importance of potassium and magnesium ions in biological phosphorus removal from wastewaters. They found that phosphorus uptake by filamentous organisms was above the amount required for the biomass synthesis (at rate similar to that of activated sludge biomass) and a substantial part of phosphorus was stored within the microorganisms as large polyphosphate polymers. Also the effects of biological phosphorus uptake depended on the presence of potassium and magnesium ions.

Schönborn *et al*. (2001) reported that the metals Ca, Mg, and K were the main components of polyphosphate granules and magnesium was found to be the key

element for the enhanced biological phosphorus removal. The addition of extra calcium and magnesium ions into the influent wastewater of an enhanced biological phosphorus removal pilot plant improved P-elimination and the quantitative ratios of these cations varied in dependence on the influent concentration of these metals. However, in another study it was suggested that magnesium and potassium were both necessary for enhanced biological phosphorus removal whereas calcium was not essential (Somiya *et al*., 1987; Rickard and McClintoc, 1992).

In a recent work, Seufferheld *et al*. (2003) reported that the volutin granules of Agrobacterium tumefaciens possess properties similar to the *acidocalcisomes* which are acidic calcium storage compartments described in several unicellular eukaryotes. Acidocalcisomes are the main storage compartments for calcium, magnesium, sodium, potassium, zinc, iron, phosphorus and amino acids for several microorganisms (De Campo *et al*., 2005). Transmission electron microscopy revealed that each intracellular granule was surrounded by a membrane and X-ray microanalysis of the volutin granules showed large amounts of phosphorus, magnesium, potassium and calcium. Calcium in the volutin granules increased when the bacteria were incubated at high extracellular calcium concentration.

Schuler *et al.* (2001) observed that increased intracellular polyP accumulation increases biomass density and improves settling characteristics (SVI). This finding is in agreement with theoretical expectations, because buoyant density is the driving force for sedimentation. The finding that increased polyP concentrations increase cell density also agrees with theoretical expectations because polyP is denser than other bacterial cell material.

Sponza (2002) have shown that phosphorus depletion caused decreases in protein and increases in polysaccharides levels resulting decreases in surface charges. Iron and phosphorus containing granule flocs were observed under phosphorus limited conditions.The results of othe Neisser staining showed that food storage granules, which are probably poly-phosphate, were present in *Nostocoida sp*. filaments from the leather industry sludges.

Thus, the findings in the literature suggests some evidence on the link between phosphorus deficiency and sludge bulking, together with cations present and phosphorus accumulation in the cells.

2.5.4. Hydrophobicity

Microbial hydrophobicity is one of the important factors influencing the surface properties and hence influencing the bioflocculation potential. Hydrophobicity is generally explained with a misleading phrase 'dislike of microbial surfaces for water'. On the contrary, all hydrophobic surfaces attract water with substantial energies, but not as strong as hydrophilic surfaces. In biological systems hydrophobic interactions are the strongest long-range non-covalent interactions and accepted as a determinant factor in microbial adhesion to surfaces (Sanin *et al*., 2003).

Researchers have shown that the hydrophobic fraction of EPS was made up only of proteins and not carbohydrates. Their results indicate that amino acids with hydrophobic side groups contribute significantly to the hydrophobicity of microbial flocs (Jorand *et al*., 1998; Liao *et al*., 2001). It was also observed that the total carbohydrate levels had a negative influence on the hydrophobicity and concluded that the presence of a large amount of hydrophilic and mainly neutral carbohydrates may be contributing to the more hydrophilic nature of sludge (Liao et al., 2001; Durmaz and Sanin, 2003).

The hydrophobicity of sludge expressed by the contact angle measurement reflects the presence of both hydrophobic and hydrophilic groups in the EPS, and is an average of the hydrophobicity of its components (Daffonchio *et al*., 1995). This statement was also supported by the findings of Liao *et al*., 2001 that the proportions of EPS components (proteins/carbohydrates and/or proteins/(carbohydrates+DNA)) were found to be more important than the quantities of individual EPS components in controlling hydrophobicity.

Allison *et al*., 1990 observed that cells that are in stationary phase of growth are more hydrophobic than cells growing exponentially On the contrary, in the study performed by Loosdrecht *et al*. (1987), the bacteria were found to become more hydrophobic at high growth rates.

Furthermore, it was also reported that food source can influence the hydrophobic character of a floc (Jorand *et al*., 1994; Boyette *et al*., 2001). The hydrophobicity of the sludge was shown to decrease as the carbohydrate to protein ratio of the feed water to microorganisms increased (Durmaz and Sanin, 2003). Research on three different types of pure culture bacteria revealed that carbon starvation does not change the hydrophobicity of bacterial surfaces, whereas, nitrogen starvation resulted a significant reduction in hydrophobicity (Sanin *et al*., 2003).

2.5.5. Surface Charge

Microbial cells, EPS, and sludge flocs carry negative charges due to the ionization of the anionic functional groups, such as carboxyl and phosphate (Horan and Eccles, 1986; Sutherland, 1990; Morgan *et al*., 1990; Keiding and Nielsen, 1997, Liu *et al*., 2004) because of the fact that EPS are anionic in the neutral pH range (Busch and Stumn, 1968; Pavoni *et al*., 1972; Morgan *et al*., 1990). In the literature, it was reported by many researchers that EPS, in both terms of quantity and quality, is crucial for the floc properties of activated sludge.

The negativity of sludge is believed to result from the carbohydrate content and the importance of the ratio of proteins to carbohydrates in determining the surface charge could be related to the unique charge properties of proteins. The amino groups in proteins carry positive charges, and can neutralize some of the negative charge from carboxyl and phosphate groups and therefore decrease the net negative surface charge of sludge flocs (Liao *et al*., 2001).

On the other hand, proteins consist of many amino acids which contain both carboxyl and amino groups. As with other organic acids, the carboxyl group ionizes in aqueous solution and the basic amino group also ionizes in solution. At certain pH

values, both the acidic and basic groups are ionized and the molecule exists as a dipolar ion (zwitterion form or hybrid ion). If such an ion were placed in an electric field, there will be a pH value at which it would migrate to neither the cathode (-) nor the anode (+). Its net charge would be zero, and this pH value is called the *isoelectric point*. Its negative and positive charges are equal, and at this point of electric neutrality, there is no tendency to move toward either pole. The net charge of the amino acid shifts with the pH. Under acid conditions, it exists in cationic (+) form, and at alkaline pH values in anionic (-) form. Each amino acid has its unique isoelectric point. For basic amino acids, e.g., lysine and arginine, this pH value is rather high -in the range of 8 to 11-, and for the acidic ones, e.g., aspartic and glutamic, it is rather low –pH 2 to 3. For those with only one carboxyl and one amino group, the isoelectric point near neutral pH (Gaudy and Gaudy, 1980).

It was also reported that the proportion of EPS components (proteins/total carbohydrates) was more important than the quantities of individual EPS components in determining the surface charge of both anaerobic and aerobic sludge flocs (Morgan *et al*.1990). Durmaz and Sanin (2003) observed that as the carbohydrate to protein ratio of the feed to the microorganisms is increased, the carbohydrate content of floc structure, and the negativity of the sludge surface also increased.

Polyvalent cations were also found to be involved in the floc structure and also had an influence on the surface charge (Steiner *et al*., 1976). Wilen *et al*. (2003) suggested that EPS showed positive correlation to negative surface charge and a negative correlation to relative hydrophobicity and flocculation ability.

Three different methods have been used in the literature to measure cell surface charge which are Zeta potential (Forster, 1968; Pere *et al*., 1993; Daffonchio *et al*.,1995; Higgins and Novak, 1997a; Nielsen and Keiding, 1998; Liu *et al*., 2001) colloid titration (Wu et al., 1982; Morgan *et al*., 1990; Finlayson *et al*., 1998, Bura *et al*., 1998; Liao *et al*., 2001; Boyette *et al.,* 2001) and dye exchange titration methods (Urbain *et al*., 1993; Boyette *et al.,* 2001;)

Zeta potential represents the potential drop in the diffuse double layer on the surface and measured based on the electrophoretic mobility in an electric field using potentiometer (Forster, 1968). Mikkelsen and Keiding (2002) reported that the zeta potential of activated sludge is in the range of -29.6±8.5 mV.

The zeta potential measured for sludge flocs under various process loading intensities (0.2-0.6 g COD/g MLSS day) did not change significantly and the average zeta potential of -24.9 mV was obtained (Chao and Keinath, 1979). They also observed no relation between zeta potential and SRT over the range of 1.1 to 11.4 days. However, Liao *et al*., (2001) reported that surface charge of the flocs decreased with increasing SRT using colloid titration method.

Contradictory findings have been reported regarding the relationships between zeta potential of sludge flocs and SVI. Some researchers obtained a positive correlation between zeta potential and SVI (Forster, 1968) while others obtained negative correlation (Magara *et al*., 1976). Moreover, Barber and Veenstra (1986), and Chao and Keinath (1979) did not observe any correlation between the two parameters.

Wilen *et al.* (2003) found that SVI increased with increasing concentrations of EPS and stated that this could be due to a stronger electrostatic repulsion between floc components as described by the DLVO theory for colloidal stability (Gregory, 1989). Similarly, Jin *et al.* (2003) obtained positive correlation between negative surface charge and SVI and explained it with DLVO theory since the presence of a net negative surface charge on floc surfaces may create repulsive electrostatic interactions which prevents close contact. However, they found that ZSV was quantitatively independent of the polymeric constituents.

CHAPTER 3

MATERIALS AND METHODS

3.1. Reactor Operation

Semi-continuous activated sludge reactors were operated using mixed culture bacteria obtained from primary sedimentation tank effluent of Ankara Central Wastewater Treatment Plant. All of the conditions as well as the feeding regime were kept constant until steady-state is reached and during the subsequent experiments.

At the beginning, three parallel reactors were operated with different calcium concentrations (5, 10 and 20 meq/L). Mixed culture reactors were fed with the synthetic feed medium. In the original feed medium, pH was maintained with phosphate buffer. Phosphate buffer formula of the (KH_2PO_4 - K_2HPO_4) was eliminated from the system in order to prevent inorganic salt precipitation in the form of $Ca_3(PO_4)_2$ which has a very low solubility product. The only phosphorus source in the medium was proteous peptone.

However, a sludge bulking situation was observed after two weeks of reactor operation and this was believed to originate from phosphorus deficiency. The only phosphorus source to the reactors was the peptone and it was able to provide only 1/20 of the stoichiometrically required value of phosphorus to sustain COD/N/P ratio of 100/5/1. Although this part was not within the original study plan; this bulking was decided to be investidated. Within this approach replicates of the bulking reactors were eliminated from the system and instead of them 3 new reactors were started under stoichiometrically adequate amount of phosphorus with previously determined calcium concentrations (5, 10 and 20 meq/L) to investigate the originally intended part of the research. The three bulking reactors were continued. Therefore,

the study had two sets of reactors operated. The bulking condition in phosphorus deficient reactors was investigated in the 1st set and the effects of calcium dose were investigated in the 2nd set of reactors.

In short, the effects of Ca ion concentration on bioflocculation were studied in both 'phosphorus deficient' and 'phosphorus suficient' conditions.

3.1.1. Phosphorus-Deficient Reactors

Three lab-scale semi continuous reactors were operated with three calcium concentrations under phosphorus deficient condition initially. The amount of phosphorus was increased to the stoichiometrically required value in two steps once the analysis of sludge was completed at each step. The calcium ion concentrations in reactors 1, 2, and 3 were 5, 10, and 20 meq/l respectively during the study.

Table 3.1.The composition of synthetic feed for phosphorus-deficient reactors

Constituent	Concentration (mg/L)
Glucose	935
Peptone	200
NH_4Cl	225
$FeSO_4.7H_20$	3.75
$ZnSO_4.7H_20$	3.75
$MnSO_4.H_20$	2.287
$NaHCO_3$	180
$MgSO_4.7H_2O$	112.5
Tris buffer	18.15
HCl	27.37

In the first step reactors were fed with severely phosphorus deficient feed water such that they received 0.6 mg/l of phosphorus corresponding to 1/20 of the stoichiometrically required value due to the addition of peptone in the feed. As it was

mentioned in the previous section, phosphate buffer was purposely eliminated from the system initially to prevent inorganic salt precipitation and, instead, tris buffer (tris – HCl) was used to adjust the pH of the reactors. Tris buffer added had a BOD_5 of 0.8 mg/L meaning that it is not easily biodegradable. The composition of the feed medium except for the studied ion (Ca) is demonstrated in Table 3.1.

The effective volume of the reactors was 2 liter with 8 days of sludge residence time (SRT) and pH was maintained at 7±0.2. The reactors were placed in a water bath in order to keep the temperature constant at 20^{o}C. The dissolved oxygen (DO) concentrations in the reactors were adjusted to be minimum as 3 mg/L at all times. For this purpose air pumps were used which also provided complete mixing of the reactors. Two hundred and fifty mL of sludge (1/8 of 2L volume) was wasted daily from the reactors since the mean cell residence time was 8 days. The schematic representation of the reactors is demonstrated in Figure 3.1.

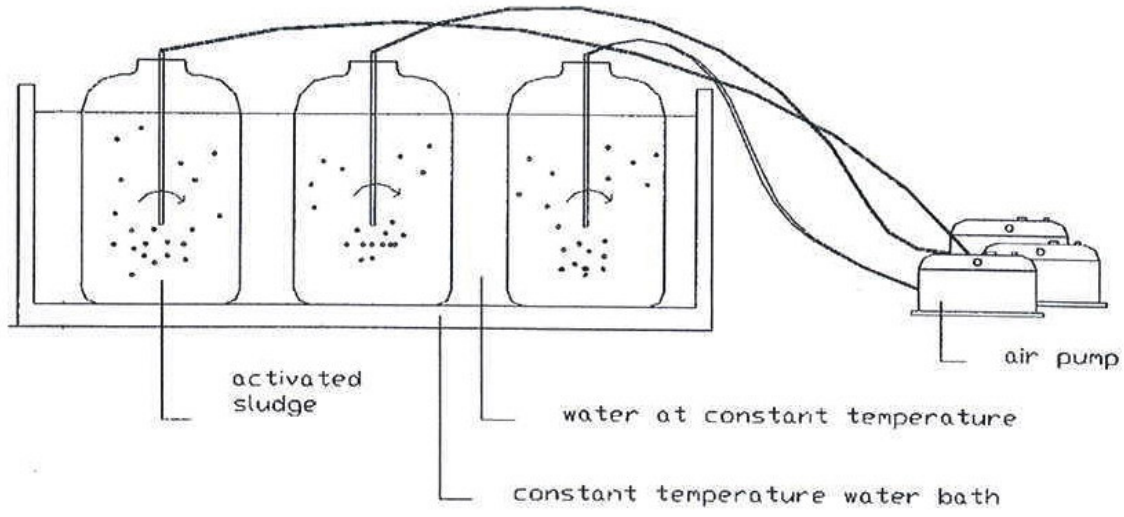


Figure 3.1. Schematic representation of reactor setup

After two weeks of reactor operation the bulking situation was recognized and this was believed to be due to the severely deficient phosphorus in the feed to the reactors. The sludge bulking was dominantly non-filamentous (viscous bulking). Therefore, this situation was decided to be further investigated in detail. The sludge bulking was then tried to be cured by increasing the phosphorus concentration of the feed water in two separate steps once enough data for each step has been collected. The concentration of the phosphorus in the feed medium was increased to 6 mg/L in the first step and 12 mg/L in the second step by adding phosphorus in the form of KH_2PO_4. Following each increase of phosphorus, reactors were run for at least 3-4 times of θ_c to achieve steady state conditions. After the second increase, the wastewater had sufficient phosphorus to supply the stoichiometrically required amount (C/N/P ratio of 100/5/1). The effect of calcium ions under phosphorus deficient condition was investigated. The phases of the study are summarized in Table 3.2.

Table 3.2. The phosphorus amount in the feed at each phase of the study

	PO_4-P concentration in the feed medium (mg l^{-1})	Corresponding COD/N/P ratio
Bulking condition	0.6	100/5/0.05
1^{st} increase of P	6	100/5/0.5
2^{nd} increase of P	12	100/5/1

At each step, reactors were allowed to reach steady state and the related chemical and physical analyses were performed as described in the following sections. In addition, microscopic examinations were done and microphotographs of sludge samples were taken at each step in order to demonstrate the changes and improvements in the structure of sludge.

3.1.2. Phosphorus-Present Reactors

Four reactors were operated throughout the study at four different calcium ion concentrations with sufficient amount of phosphorus. The COD/N/P ratio of the feed

was 100/5.9/0.83. Control reactor (reactor 4) had 0.27 meq/L Ca ion concentration and reactors 5, 6 and 7 had 5, 10 and 20 meq/L calcium concentration respectively.

Control reactor was used as basis for observing the effects of increasing calcium concentration. The reason of maintaining the calcium concentration of control reactor at 0.27 meq/L but not zero was to supply the minimum metabolically required amount of calcium ion for the proper reproduction of microorganisms.

Unlike the phosphorus-deficient reactors, the reactors were fed with another type of peptone (biosate peptone) having high phosphorus content (%3.9) in this set. As the phosphorus was in organic form, this would prevent calcium-phosphate salt precipitation. No buffer was used to adjust the pH and pH of the reactors was 7.7 ± 0.3. The composition of the feed medium for control reactor is given in Table 3.3.

Table 3.3. Composition of the synthetic feed medium for control reactor

Constituent	Concentration (mg/L)
Glucose	163.54
Biosate Peptone	942.5
NH_4Cl	225
$MgSO_4.7H_2O$	112.5
$FeSO_4.7H_2O$	3.75
$ZnSO_4.7H_2O$	3.75
$MnSO_4.7H_2O$	3.75
$CaCl_2$	15
$NaHCO_3$	180

The other operating conditions were the same as the phosphorus-deficient reactors. Similar reactors with the same schematic representation as it is demonstrated in Section 3.1.1. were used in this part, too.

3.1.3. Steady-State Determination

The reactors containing sufficient amount of phosphorus (reactors 4, 5, 6 and 7) were all brought to steady sate by holding the above listed conditions constant. However, because of the fact that the phosphorus deficient reactors (reactors 1, 2 and 3) bulked at the beginning of operation, achievement of steady state was very difficult. Therefore their first series of analysis were conducted at this situation. Then they were allowed to reach steady state after the first and second increase of phosphorus amount of the feed without any problems of attaining the steady state.

It is stated in the literature that at least 3 SRT is required for microorganisms to adopt themselves to new conditions (Forster and Dallas-Newton, 1980). The steady state determination was achieved by measuring the mixed liquor suspended solids (MLSS), and mixed liquor volatile suspended solids (MLVSS) concentrations after a time period of minimum 4 SRT. Obtaining the relatively constant results (about less than 15% variation) for these parameters during 2 or 3 consecutive measurements indicated that steady state had been reached. Then the following analyses were performed.

3.2. Analyses Conducted Under Phosphorus-Deficient Conditions

The analyses conducted for phosphorus-deficient reactors at each series include carbohydrate measurement, phosphate phosphorus (PO_4-P) determination, MLSS and SVI determination and taking microphotographs of the sludge samples. The response of microorganisms at different phosphorus concentrations to increasing calcium ion concentration in the feed were determined based on the results of these analyses.

3.2.1. Carbohydrate Analysis

A pretreatment procedure developed by Yurteri (1982) was applied to the bacterial culture from the phosphorus-deficient reactors in order to burst the cells and extract their carbohydrate contents before carbohydrate analysis. For this purpose, 5 mL

sludge taken from the reactor medium was centrifuged at 3500 rpm for 15 minutes and the supernatant was discarded. Then 25 ml 1N NaOH was added into the tube containing the bacterial cells and vortexed for complete mixing. This mixture was boiled until the cells were burst and then centrifuged again. The centrates were used for carbohydrate analysis. Carbohydrate analysis was conducted according to phenol sulphuric acid method which is described in detail in section 3.3.1.2.1.

3.2.2. Phosphate-phosphorus (PO_4-P) Analysis

Completely mixed sludge sample of 50 mL from each reactor was centrifuged and the supernatant was discarded. Then the solid part was completed to 50 mL with distilled water and this sample was used for the analysis. PO_4-P determination was conducted based on the Standard Method 4500-P named as Automated Ascorbic Acid Reduction Method determined by APHA (1989).

3.2.3. Microphotographs

Sludge samples obtained from the completely mixed reactor medium of the phosphorus-deficient reactors were examined under microscope and their photographs were taken with necessary magnification using a Leica DFC 280 model microscope.

3.3. Analyses Conducted Under Non-Limited Phosphorus Conditions

After the reactors reached steady state, chemical, surface chemical and physical characteristics of the sludge samples were determined in order to find out the effect of calcium ion concentration on activated sludge properties.

3.3.1. Determination of Chemical Characteristics of Activated Sludge

Chemical characteristics of activated sludge are known to be closely related to the extracellular polymeric substances (EPS). One of the purposes of the study was to test the differences in the characteristics of extracellular polymers grown at different

calcium concentrations. For this reason, after the reactors reached steady state, the EPS were extracted from activated sludge floc structure and then analyzed for its carbohydrate and protein content.

3.3.1.1. Polymer Extraction by Cation Exchange Resin (CER) Method

The extraction of EPS from the floc structure was performed using a cation exchange resin (CER) as first suggested by Frolund *et al.* (1996). Dowex 50x80, Na^+ form (20-50 mesh) strongly acidic cation exchange resin was supplied from Fluka and a method which was similar to the one utilized by Frolund *et al.* (1996) and fully developed by Durmaz and Sanin (2001) was used.

Before the extractions were performed, MLVSS value of each sludge sample was measured and a dose of 100 g CER/ gVSS was used for each sludge sample based on the previous study (Durmaz and Sanin, 2001). The separately weighed cation exchange resins for each reactor were put into glass beakers and washed with a phosphate buffer saline (PBS), composition of which is given in Table 3.4, about 2 hours. The reason of washing was to prevent leaching from the CER and to eliminate subsequent interferences during chemical analysis. When washing was complete, CER was filtered using millipore filter and dried at room temperature until the next day (Frolund *et al.* 1996).

Table 3.4. Composition of phosphate buffer saline solution

Constituent	Concentration (g/L)
NaCl	4
KCl	0.1
KH_2PO_4	0.06
Na_2HPO_4	0.455

Extracellular polymer extraction procedure was conducted on daily wasted sludges. 200 mL of wasted sludge from all of the reactors was centrifuged at 3500 rpm for 15 minutes. After centrifuging, the centrate was discarded and the pellet was

resuspended to 200 mL using phosphate buffer saline (PBS) solution in a jar. Then the dried CER from the previous day which was weighed for that sludge sample was added into the resuspended sludge in PBS solution. Apart from this, two controls were prepared for each reactor. The first control was a sludge sample from the same reactor with no CER addition (sludge-control). The purpose of the first control was to find out if there were any polymers extracted by only the stirring effect.The second control was CER in PBS solution containing no sludge in it (CER-control). During the extraction only one CER-control was used for all reactors since the contribution from the CER itself had been found almost zero in a previous study (Durmaz, 2001). After the same procedure was applied for each reactor, the jars (extraction beakers) were stirred at a standard jar test apparatus for 5 hours at a speed of 120 rpm. This time duration was chosen based on the study performed by Durmaz, (2003).

3.3.1.2. Composition of Extracellular Polymeric Substances

EPS was released to the medium as a result of cation removal from floc structure during stirring the sludge samples with CER. After 5 hours mixing, the jars were left to settle down for about half an hour. Then the supernatants were centrifuged for 10 minutes and the centrates were used for carbohydrate and protein analyses.

3.3.1.2.1. Carbohydrate Analysis

Carbohydrate content of EPS in the centrates of the samples were measured based on phenol sulphuric acid method of Dubois *et al.* (1956) using alginate as the standard.

First, 2 mL from each sample was put into separate test tubes, and then 50 µL from 80% (w/w) phenol and 5 mL sulphuric acid were added respectively. Phenol was prepared by adding 20 grams of distilled water to 80 grams of redistilled reagent grade phenol and sulphuric acid was reagent grade at 98 %. The mixtures were allowed to stand at room temperature for 10 minutes and then vortexed and put into an incubator at 30 ^{o}C for 15 minutes. The absorbance of the characteristic yellow-orange color was measured by Milton Roy Company Spectronic 20D at 480 nm

wavelength. Blanks were prepared by substituting distilled water for EPS samples. The amount of carbohydrates was determined by reference to a standard curve (Appendix A) previously constructed for alginate. In order to calculate the amount of carbohydrate belonging to EPS, the carbohydrate concentrations of CER-control and sludge-control samples were subtracted from the value of CER-sludge sample. All of the measurements were done in triplicates.

3.3.1.2.2. Protein Analysis

Protein type of polymers in each EPS extraction set were determined using folin-ciocalteu protein measurement method designated by Lowry *et al.,* (1951). Bovine Serum Albumin from Sigma (A-7906) was used as a standard for the preparation of the calibration curve.

Four reagents were used in the analysis named as reagent A, B, C, and D. Reagent A included 2 % (w/v) sodium carbonate in 0.1 N NaOH. Reagent B was composed of 1 % w/v sodium potassium tartarate in 0.5 % (w/v) cupric sulphate. Reagent C contained 1ml of Reagent B and 49 ml of Reagent A. Reagent D included Folin-Ciocalteu's phenol reagent which was diluted by the ratio of 10:9 with distilled water.

Samples obtained from the centrates of CER extraction at 0.6 mL volume were put into test tubes. Three mL of Reagent C was added and the mixture was allowed to stand for 10 minutes at room temperature. Then, 0.3 mL of Reagent D was added and the mixture was vortexed immediately afterwards. The constituents in the test tube were left to stay at room temperature for 30 minutes. The same procedure was applied to all CER sets in triplicates. The intensity of the blue color was read at 750 nm using Milton Roy Company Spectronic 20D against a reagent blank. Protein concentrations were calculated based on the calibration curve given in Appendix A. The protein concentration of CER-control and sludge-control samples were subtracted from the value of CER-sludge sample to calculate the amount of protein in EPS as in the case of carbohydrate measurement

3.3.1.3. Calcium Ion Concentration in Sludge

Calcium ion concentration in each sludge sample at steady state was determined based on microwave assisted digestion procedure of sludge as described by (Özsoy *et al*. (2005). Prior to the application of the method, the wasted sludges were dried at 105^{o}C for 24 hours. Then at least 0.25 g dried sludge was weighed for each reactor and put into different teflon vessels of Berghof speedwave MWS-2 microwave digester. The digestion procedure was conducted according to a program using 5 mL HNO_3 and 5 mL HF combination. The program consists of three stages, the details of which are demonstrated in Table 3.5. The supernatants obtained from the reactors were also digested with the same method. After the digestion process, the sludge samples and supernatants were put into small glass beakers and allowed to boil until 3-5 ml sample is left. Then the remaining samples were diluted and the calcium ion contents of the diluted samples were measured using ATI Unicom 929 Atomic Absorption Spectroscopy.

Table 3.5. Stages of microwave digestion process

Program Stages	Time (min)	Temperature (^{o}C)	Power (W)
Stage 1	40	200	800
Stage 2	25	100	400
Stage 3	1	20	40

3.4. Determination of Surface Chemical Characteristics of Activated Sludge

3.4.1. Hydrophobicity

The contact angle measurements and the Microbial Adhaesion to Hydrocarbons (MATH) tests are the most commonly used methods to evaluate hydrophobicity. The MATH test that was developed by Rosenberg *et al*. (1980) is used in this research in order to measure the surface hydrophobicity of microorganisms. The MATH test exploits the tendency of various bacterial strains possessing hydrophobic surface characteristics to adhere to liquid hydrocarbons (e.g. n-hexadecane, n-

octane) (Rosenberg *et al*., 1980). In this study, n-Hexadecane was used as the hydrocarbon phase.

Before the MATH test was applied, sludge samples from each reactor were washed twice and resuspended with phosphate buffer solution (PBS) by centrifuging at 3500 rpm for 5 minutes. Composition of PBS is given in Table 3.4. After the sludge samples were vortexed for a minute, 5 mL of bacterial suspension was taken and the absorbance (optical density) was adjusted to 0.4 at 600 nm using the Milton Roy Company Spectronic 20D by making the necessary dilutions with phosphate buffer solution. This constitutes the initial optical density. After adjusting the absorbance, at least 5ml bacterial suspension was transferred to a clean test tube and 0.5 mL n-hexadecane was added. The mixture was homogenized for about 2 minutes using a vortex mixer and the hydrocarbon phase was allowed to separate completely for 15 minutes. Following 15 minutes, the liquid part that remained under the n-hexadecane phase was taken with the help of a Pasteur pipette and its absorbance was measured as the final optical density. Then the percentage of adhesion to hydrocarbons was calculated as follows:

$$Hydrophobicity(\%) = (1 - \frac{OD_{final}}{OD_{initial}}) * 100 \quad \text{.....(3.1)}$$

3.4.2. Zeta Potential

Zeta potential corresponds to the potential measured at the surface enclosing the fixed layer of ions attached to the particle (Tchobanoglous and Burton, 1991). Zeta potentials of the sludge samples were measured using Zeta Sizer Nano series ZS90. Before the measurements, completely mixed sludge samples were sonicated in a water bath in plastic centrifuge tubes and then diluted up to the range that the device could detect the zeta potential on sludge particles. Phosphate buffer saline (PBS) composition of which is given in Table 3.3 was used for dilutions.

3.5. Determination of Physical Characteristics of Activated Sludge

Calcium concentration of the feed is expected to affect the composition of EPS matrix and hence the flocculation ability which in turn determines the sludge characteristics. With the purpose of defining a relationship between calcium ion concentration and the physical properties of activated sludge, the following tests were performed: viscosity and the rheological analysis, specific resistance to filtration (SRF), sludge volume index (SVI), zone settling velocity (ZSV), and supernatant turbidity.

3.5.1. Viscosity

In order to determine the effect of calcium concentration on sludge rheology, the rheological behaviour and apparent viscosity of sludge samples were measured using a rotational viscometer having a cylindrical spindle (Brookfield, LVDVII+, with ultra low viscosity adapter, Figure 3.2).

Figure 3.2. Brookfield Viscometer

Since sludge rheological behaviour is dependent on both solids concentration and the shear rate applied (non-Newtonian behaviour) a series of analyses at different solids concentration and shear rates is necessary to be conducted. Sludge samples of five different suspended solids concentrations were prepared for each reactor so as to obtain a relationship between sludge viscosity and solids concentration. Sludge's own supernatants after settlement were used for dilutions in preparation of the different solids concentrations. Due to the non-Newtonian flow property of sludge, viscosity is dependent on the shear rate applied. Hence, viscosity measurements were performed also at six different shear rates ranging from 1.83 to 73.38 sec^{-1} at 26±1^{o} C. Then apparent viscosities were calculated by taking the ratio of shear stress to shear rate at corresponding shear rates. For all of the reactors, these viscosity values were plotted against five different solids concentrations of each reactor and at a fixed solids concentration and shear rate, viscosity values of each reactor were compared. Also the shear stress shear rate relationships were examined to investigate the degree of non-Newtonian behavior.

3.5.2. Specific Resistance to Filtration

Filterability characteristics of the sludge samples were determined in terms of Specific Resistance to Filtration (SRF) test as described by Vesilind (1979). A 50 mL of sample from each reactor was filtered under vacuum into a graduated cylinder. At every 5 mL volume interval of filtrate withdrawn, the time was recorded. The tests were conducted at 21 in Hg vacuum pressure and Whatman 42 filter paper was used during filtrations. Following the filtrations, the weight of the sludge cake deposited on the filter paper was determined by gravimetric analysis. Time/volume (t/V) versus volume (V) graphs were plotted for each reactor using the previously recorded time and volume values. Specific resistance to filtration was then calculated from the slope of this graph using the below expression:

$$r = \frac{2PA^2b}{\mu w} \quad \text{.....(3.2)}$$

where;

r = Specific resistance to filtration, m/kg

P = Pressure difference, N/m^2

A = Filtration area, m^2

b = Slope of the t/V versus V graph, s/m^6

μ = Viscosity of filtrate, $N.s/m^2$

w = Weight of dry cake solids per unit volume of filtrate, kg/m^3

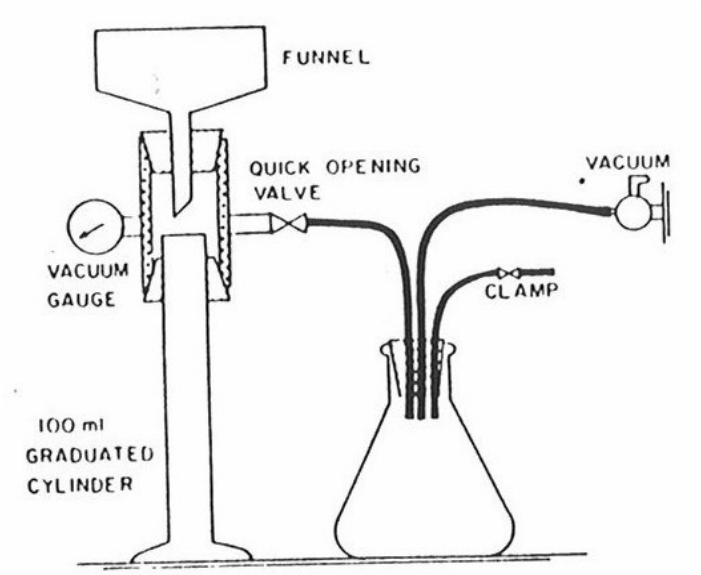


Figure 3.3. Buchner funnel apparatus for measuring specific resistance

3.5.3. Sludge Volume Index (SVI)

Sludge volume index (SVI) which is a quick interpretation of settling behavior of sludge was determined by placing a 1L of completely mixed sludge sample into a graduated cylinder and measuring the settled volume after 30 minutes. SVI value was calculated using the following formula:

$$SVI = \frac{\text{Volume of sludge after 30 minutes settling (mL/L)} * 1000}{\text{Suspended solids concentration (mg/L)}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3.3)$$

3.5.4. Zone Settling Velocity

Zone settling velocity was measured pouring a well mixed sludge sample into a 1 L graduated cylinder and allowing it to settle. Time was recorded at every 20 ml volume intervals of solid-liquid interface on the cylinder corresponding to 0.5 cm height at each interval. Data was collected for about 1 hour in order to obtain sufficient data to assure that suspension was exposing a constant zone settling velocity. For the purpose of finding out the effects of solids concentration on settleability, the experiment was conducted on three different concentrations of each sample. When all of the measurements were completed, interface height (cm) versus time (s) graph was plotted for each concentration. A sample graph is demonstrated in Figure 3.4 for reactor 5 at its MLSS value. Then, ignoring the initial shoulder (reflocculation period), a straight line was drawn such that the slope of the line was the zone settling velocity in cm/s. Subsequently, zone settling velocity (cm/s) versus solids concentration (mg/L) graph was plotted and at a constant concentration for each reactor and the ZSV values of different reactors were compared.

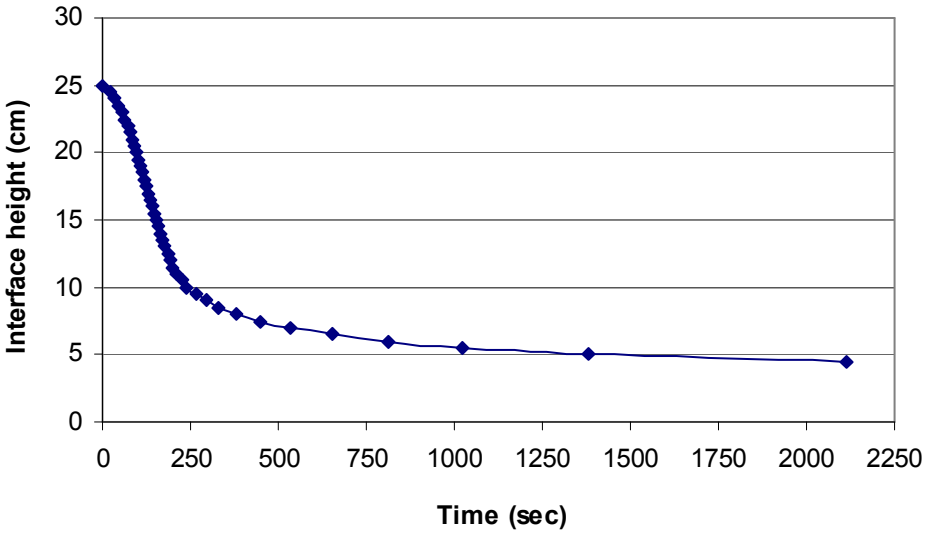


Figure 3.4. Interface height vs. time graph for reactor 5 (Ca = 5 meq/L) at MLSS = 2760 mg/L

3.5.5. Turbidity

All the sludge samples were allowed to settle for 60 minutes. Following 60 minutes settlement residual turbidities were measured in Nephelometric Turbidity Unit (NTU) using Hach Turbidimeter 2100A which was previously calibrated with standard turbidity solutions.

3.6. Other Measurements

3.6.1. Ammonia and Total Kjeldahl Nitrogen (TKN) Analyses

Ammonia and Total Kjeldahl Nitrogen (TKN) analyses of the feed wastewater were conducted according to the Standard Method 4500 (APHA, 1989). In both of the experiments 25 mL of sample was used for the analyses.

3.6.2. MLSS, MLVSS, COD, pH and DO

Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) measurements were conducted according to Standard Method for the examination of waster and wastewater method number 2540 (APHA, 1989).

COD measurements were conducted according to EPA approved method as described in Hach Water Analysis Handbook (1989). Closed reflux colorimetric method was used and the COD values were measured with Hach DR2000 spectrophotometer.

pH of the reactor medium and other necessary solutions were measured using Jenway 3010 pH meter.

Dissolved oxygen was measured via YSI model 51B DO Meter.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Effect of Calcium Ion Concentration in Phosphorus Deficient Reactors

This phase of the study was conducted under three stages depending on the phosphorus concentration of the feed media as summarized in Table 3.1 in Section 3.1.1. Following the initial assessments and demonstration of bulking situation with 1st stage of experiments, the concentration of phosphorus in the feed medium was increased stepwise first to 6 mg l^{-1} and then to 12 mg l^{-1}. The increased phosphorus was provided in the form of KH_2PO_4 salt to the reactors.

4.1.1. Steady State Conditions

Three phosphorus deficient reactors were operated under three different calcium concentrations. The calcium ion concentrations in reactors 1, 2 and 3 were 5, 10 and 20 meq/L, respectively. The reactors were laboratory-scale semi continuous activated sludge reactors having 2 L working volume and 8 days of sludge residence time (SRT). Mixed culture bacteria were grown in the reactors and fed with the synthetic feed medium composition of which is defined in Table 3.1. Temperature (20^0C) and pH (7) were kept constant to eliminate the effects of these parameters on the system and the DO concentration was kept above 3 mg l^{-1}. The C/N ratio of the feed to the reactors was 20 in terms of the ratio of COD to TKN.

After each increase of phosphorus, reactors were run for at least 4 times of SRT to achieve steady state conditions since in the literature it is reported that sludge requires about 3 SRT stabilization period before any response to the new conditions

can be reflected (Forster and Dallas-Newton, 1980). With our observations and also according to MLSS and MLVSS values it was not possible to demonstrate a steady state in the first stage of the study. Since the sludge was severely bulking it was not possible to settle the sludge for proper reactor maintenance and operation. So the analyses of the sludge were conducted without satisfying the 'steady state' criteria.

4.1.2. Physical and Chemical Properties of Activated Sludge at Different Calcium Ion Concentration under Severely Phosphorus Deficient Condition

Avoiding phosphorus from the feed medium of the reactors resulted in a serious sludge bulking problem. The only source of phosphorus in the feed medium was the peptone used and it alone could not supply the required amount according to the required C/N/P ratio of the system. The analysis of the feed revealed that only about 1/20 of the stoichiometrically required amount of phosphorus was supplied to the microorganisms. The COD/N/P of these reactors were 100/5/0.05. Hence the reason of bulking was attributed to the phosphorus deficient condition in the reactors which was also confirmed by previous investigators (Ericsson and Eriksson, 1988; Jenkins *et al*., 1993; Sponza, 2002).

A series of experiments were conducted in order to demonstrate the characteristics of sludge, the results of which are given in Table 4.1. As it is seen from the table, the SVI values are extremely high for all the reactors indicating the severity of bulking. In the literature the critical value for SVI is taken as 150, above which the sludge is said to be bulking. Moreover, there are not any calcium ion concentration dependent differences observed for the reactors studied in bulking situation.

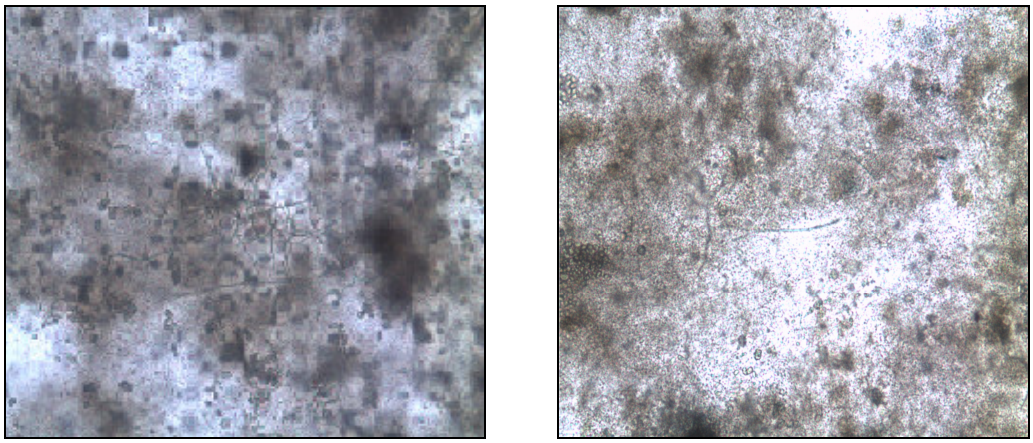
When the microphotographs of the sludge samples were examined it was seen that sludges from all of the reactors exhibited viscous bulking with no obvious filamentous microorganisms present (Figure 4.1. a, b and c). On the contrary, in a parallel study performed under the same conditions exactly, except for the cation type, which was magnesium instead of calcium, excessive growth of filamentous bacteria was observed from the microphotographs (Turtin *et al*., 2005).

Hence, depending on these results it can be concluded that phosphorus deficiency in the wastewater causes sludge bulking, however, it is the type of cation that determines the type of bulking whether it is viscous or filamentous. In this research, microorganisms grown under phosphorus deficient and calcium rich conditions were observed to cause viscous bulking independent of the dose of calcium fed to the reactors.

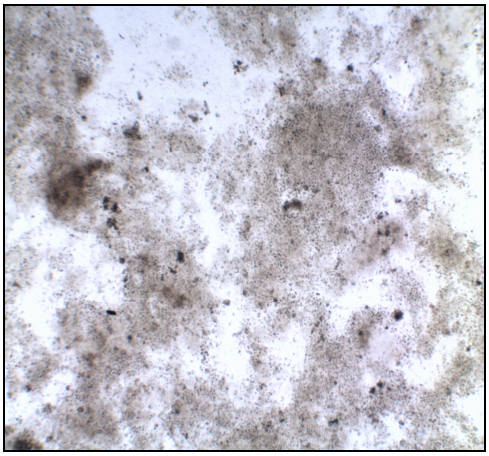
Table 4.1. Results of the experiments during phosphorus deficient operation

Parameter	Reactor 1	Reactor 2	Reactor 3
Calcium in the feed (meq/L)	5	10	20
PO_4-P (mg/L) in the sludge	0.375	0.316	0.191
SVI	1081	995	308
MLSS (mg/L)	840	1005	1135
Sludge Carbohydrate Conc. (mg/L)	161.7	100	148
Carbohydrate (as the % of MLSS)	19.4	10	13
PO_4-P (as the % of MLSS)	0.045	0.031	0.017

To confirm the viscous bulking, which is typically evident at high carbohydrate loadings, sludge total carbohydrate concentrations were measured. If viscous bulking is the mechanism, then the carbohydrates are expected to be at least as 20% in sludge composition (Jenkins *et al*., 1993). Among all the reactors 1, 2, and 3, the measured sludge carbohydrate concentration from reactor 1 only was about 20% of the overall sludge mass. The other reactors had 10 and 13 %, which are not overly high values as expected for viscous bulking sludges (Table 4.1). However, these results still indicate that the sludges are rich in terms of carbohydrate content. The remarkable observation providing a better proof of viscous bulking indeed was the microphotographs as discussed above and the SVI values.



a. **b.**



c.

Figure 4.1**.** Sample microphotographs when COD/N/P is 100/5/0.05 for **a.** Ca = 5 meq/L **b.** Ca = 10 meq/L and **c.** Ca = 20 meq/L at 10X magnification

The other important finding obtained from these analyses is that the reactors operated under phosphorus limited situation had very low MLSS values. When the same reactors were operated at non-limiting phosphorus concentration they were able to produce MLSS values around 2500-3000 mg/l at steady state which are presented in the second part of this chapter. However the MLSS values measured were around 1000 mg/L in this case (Table 4.1). The low MLSS values could be explained by the fact that the cells were growing under strictly limited phosphorus condition as the MLSS production was determined by the limiting nutrient in the feed. In this feeding pattern both the carbon and nitrogen were in excess and probably were not used for cell synthesis. It is known that microorganisms produce EPS when the amount of carbon in the media is more than the needed amount for growth and maintenance. The produced EPS, which is negatively charged, itself requires an agent to bind onto surface of microorganisms, and to each other. In this respect, calcium ions seem to fulfil this requirement. The role of calcium in the binding of EPS was also verified by many authors in the literature (Forster and Lewin, 1972; Eriksson and Alm, 1991; Bruus et al., 1992; Murthy et al., 1998; Higgins and Novak, 1997abc; Keiding and Nielsen, 1997; Sanin and Vesilind, 1996 and 2000).

In this study, since there was enough calcium in the growth media, as EPS was produced, polymers were bound to the surface of flocs and to each other. By this way, when the floc matrix was completed over time, excess production of EPS under conditions when calcium was in excess, ended up producing viscous bulking sludge. Furthermore, the presence of calcium ions in the growth media might also have some stimulating effect on the production of EPS which is also in parallel with our findings in the 2nd phase of the study which is discussed in the 2nd part of this chapter.

4.1.3. Physical and Chemical Properties of Activated Sludge at Diferent Calcium Ion Concentration When the Phosphorus is Increased to Half of the Stoichiometrically Required Value

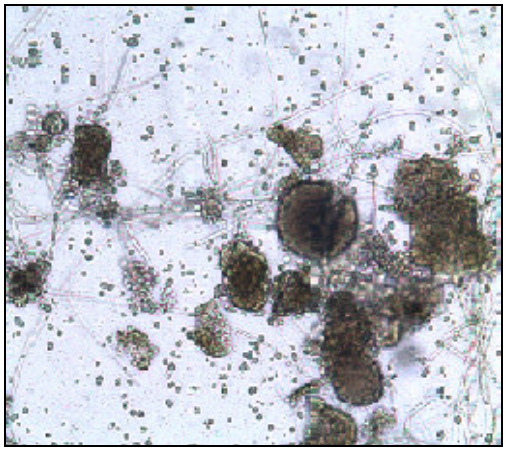
Right after the demonstration of bulking situation with the 1st series of experiments, the amount of phosphorus in the feed medium was increased by 10 folds from 0.6 to

6 mg/L corresponding to the COD/N/P ratio of 100/5/0.5 which is equivalent to the half of the stoichiometrically required value by the microorganisms. The reactors were allowed to reach steady state for 6 weeks and then the 2nd series of experiments were conducted in order to identify the effect of increasing phosphorus amount of the feed on sludge bulking at different calcium concentrations.

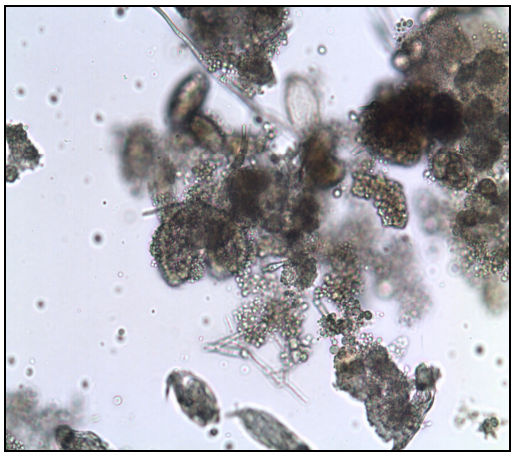
Table 4.2. Results of the experiments during COD/N/P is 100:5:0.5

Parameter	Reactor1	Reactor 2	Reactor 3
Calcium in the feed (meq/L)	5	10	20
PO_4-P (mg/L) in the sludge	2.3	3	2.4
SVI	28	38	48
MLSS (mg/L)	2375	2530	2480
MLVSS (mg/L)	2190	2270	2210
Sludge Carbohydrate Conc. (mg/L)	146.3	339	239
Carbohydrate (as the % of MLSS)	6.2	13.4	9.6
PO_4-P (as the % of MLSS)	0.097	0.119	0.097

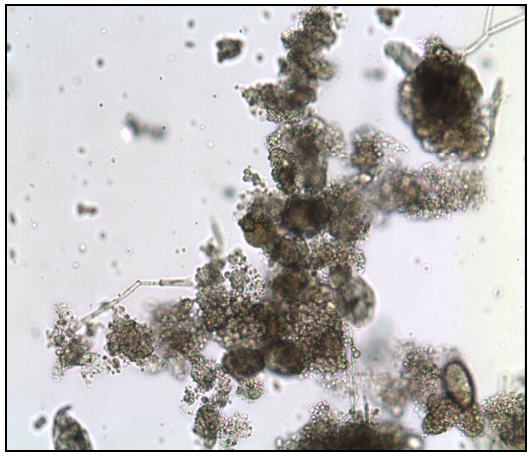
As can be seen in Table 4.2, the settling characteristics of the sludge samples improved as the phosphorus concentration in the feed was increased. SVI values of all of the three sludges fell sharply to low levels. In agreement with the settlement results, microscopic examinations showed that the floc structure for all the reactors changed significantly (Figure 4.2. a, b and c). Some filamentous microorganisms were visible in the flocs indicating a healthier floc formation and the viscous bulking problem disappeared to a great extent. Since all the other system parameters were kept constant, it is concluded that the reason for this improvement is the increase in the amount of phosphorus in the feed water.



a.



b.



c.

Figure 4.2. Sample microphotographs when COD/N/P is 100/5/0.5 for **a.** Ca = 5 meq/L **b.** Ca = 10 meq/L and **c.** Ca = 20 meq/L at 10X magnification

Parallel to the increase in the amount of phosphorus in the reactor feed medium, the percentage of PO_4-P in the total sludge mass also increased as indicated in Table 4.2. Moreover, MLSS concentrations increased close to the expected values of a typical balanced reactor for all of the reactors. When considering the results of 2nd stage of experiments, a general decrease in carbohydrate content of sludge was expected since the feed media was better balanced in terms of C/N/P. Examining Table 4.2 it is seen that the amount of carbohydrate as the percentage of the total mass (MLSS) decreased for reactors 1 and 3 while it increased for reactor 2. No valid reason for this increase of sludge carbohydrate concentration could be identified because of the fact that the amount of carbohydrate in the feed medium remained the same as in the first step. However, based on the SVI data it can be said that the increase in carbohydrate concentration of reactor 2 did not have a negative impact on the settling characteristics of the sludge. Findings of the experimental analyses (Table 4.2) together with the microphotographs (Figure 4.2. a, b and c) indicate that as in the case of severely phosphorus deficient condition sludge properties were independent of the calcium concentrations used.

4.1.4. Physical and Chemical Properties of Activated Sludge at Different Calcium Ion Concentration When the Phosphorus is Increased to the Stoichiometrically Required Value

When the 2nd series of experiments were completed, the amount of phosphorus in the feed medium was increased to 12 mg/L which is the stoichiometrically required value corresponding to COD/N/P ratio of 100/5/1 that satisfies the proper feed media for production of microorganisms. The reactors were operated for 5 weeks until they reached steady state and then the 3rd series of analyses were conducted (Table 4.3).

When the results are examined it is seen that all reactors settled down to a steady level of carbohydrates (4 – 7 %). On the whole, the general trend of carbohydrate concentration was in the decreasing direction as expected. If the bulking and steady state conditions are compared, the amount of sludge carbohydrate decreased from 19% to 6.8% for reactor 1, from 10% to 5.5% for reactor 2 and from 13% to 3.1 % (as % of MLSS) for reactor 3.

Table 4.3. Results of the experiments during COD/N/P is 100/5/1

Analysis	Reactor 1	Reactor 2	Reactor 3
Calcium in the feed (meq/L)	5	10	20
PO4-P (mg/L) in the sludge	11.4	13	12.1
SVI	45	40	41
MLSS (mg/L)	2530	2830	2740
Sludge CarbohydrateConc. (mg/L)	173.5	156.8	100
Carbohydrate (as the % of MLSS)	6.8	5.5	3.6
PO_4-P (as the % of MLSS)	0.451	0.459	0.442

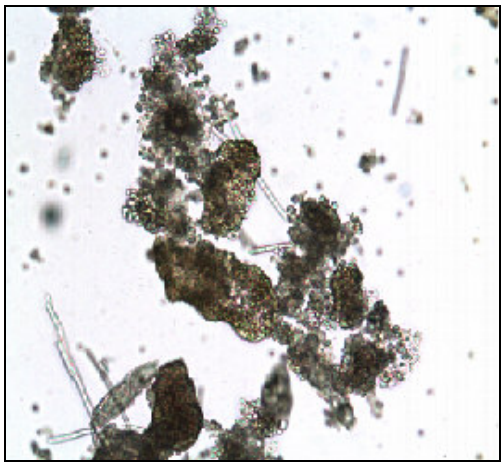
The SVI data clearly indicates that the bulking situation that occurred due to phosphorus deficiency has been completely cured in this third stage of the study. Based on the results obtained from 1st, 2nd and 3rd series of experiments it is evident that considerable amount of phosphorus was accumulated in the sludges of the three reactors. According to the phosphorus concentration data, from the 1st stage to the 2nd stage, the increase in phosphorus concentration of the feed medium was 10 times. This resulted in approximately 10 times increase in the phosphorus measured in sludge of 2nd stage of reactors compared to the 1st stage (Table 4.1 and 4.2). However, when the sludge phosphorus was normalized with respect to MLSS in the reactors; data in Tables 4.1 and 4.2 show that there is not a 10 times increase in the values as expected; rather the increase is around 4 times. The reason behind this is thought to be the sharp increase in MLSS concentration from stage 1 to stage 2.

When the reactors were passed to stage 3 from stage 2, the feed phosphorus concentration was increased only twice. However Tables 4.2 and 4.3 show that sludge phosphorus concentration was increased by about 5 times and the PO_4-P concentrations normalized with respect to MLSS also increased by an average of 4.3 times. Both of these increases indicate that there is accumulation of phosphorus beyond the metabolically required amount by the microorganisms. This situation is much clearer in the last stage even though it is also believed to happen but offset by MLSS normalization in the 2nd stage of experiments as well. Hence these results

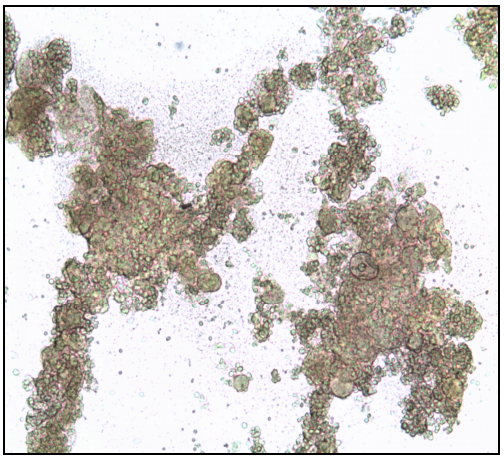
altogether indicate significant accumulation of phosphorus beyond the stoichiometric amount provided to the reactors.

In agreement with the experimental results, microscopic investigations have shown some granular structures in the microphotographs (Figure 4.3. a, b and c). It is known that holding cells for a certain time under phosphorus starvation followed by exposure to phosphorus can lead to a great accumulation of polyphosphate granules. The primary purpose of the stored polyphosphate in most bacteria is a phosphorus source for periods of phosphorus starvation (Wanner, 1994). These structures observed under microscope are thought to be polyphosphate granules, mentioned by many other authors in the literature (Schönborn *et al*., 2001; Khoshmanesh *et al*., 2002; Seufferheld *et al*., 2003; DeCampo *et al*., 2005). Machnicka *et al*. (2005) also showed similar polyphosphate structures visible by bright field microscope.

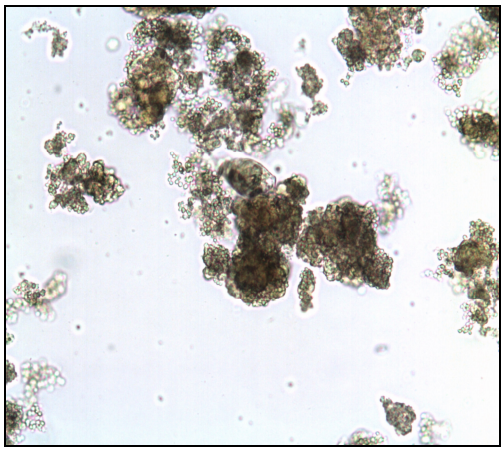
Having cured the viscous bulking problem by increasing the phosphorus concentration in two stages, this first phase of the study was terminated. In the second phase of the study, the experiments related to the investigation of calcium ions and bioflocculation of activated sludge, as it was originally intended to be studied, were carried out.



a.



b.



c.

Figure 4.3. Sample microphotographs when COD/N/P is 100/5/1 for **a.** Ca = 5 meq/L **b.** Ca = 10 meq/L and **c.** Ca = 20 meq/L at 10X magnification

4.2. Effect of Calcium Ion on Sludge Properties

In this part of the study the effects of calcium ion concentration on the physical, chemical and surface chemical properties of activated sludge have been investigated in a non-limiting phosphorus condition. New reactors were started from the beginning with the phosphate salts eliminated. Phosphorus was provided with the addition of a phosphorus rich peptone in the feed. Composition of the synthetic feed medium to the reactors is given in Table 3.3 in section 3.1.2.

4.2.1. Steady State Conditions

Four semi-continuous lab-scale activated sludge reactors were operated at four different calcium concentrations. Reactor 1 was the control reactor having 0.27 meq/L calcium concentration which was considered the minimum required amount for proper production of microorganisms. Reactors 2, 3 and 4 were operated under 5, 10 and 20 meq/L calcium concentrations respectively. Temperature of the reactors were 20^0C, pH was 7.7 ± 0.3. DO concentration was kept above 3 mg l^{-1}. The C/N ratio of the feed to the reactors was 17 in terms o COD/TKN.

The reactors were first brought to steady state before the experimental analyses. The steady state condition was monitored by measuring MLSS and MLVSS values (Table 4.4). Also it was waited for at least 6 SRT in order to be on the safe side for steady state. However, after this period of time, the SVI of control reactor (Ca = 0.27 meq/L) was 213 indicating a bulking condition which makes it very difficult to reach steady state. The high variations in the MLSS data also supports this situation (Table 4.4). Thus, the relevant experiments were conducted after steady state has been reached for the other three reactors except from the control reactor. The results are presented and discussed in the foregoing sections. Effluent COD values are presented in Figure 4.4.

Table 4.4 Evaluation of steady state conditions at non-limiting phosphorus condition

Dates	Control Reactor (Ca=0.27 meq/L)			Reactor 1 (Ca=5 meq/L)			Reactor 2 (Ca=10 meq/L)			Reactor 3 (Ca=20 meq/L)		
	MLSS	*MLVSS*	*ratio*	*MLSS*	*MLVSS*	*ratio*	*MLSS*	*MLVSS*	*ratio*	*MLSS*	*MLVSS*	*ratio*
04.07.2004	2215			2595			2115			2510		
08.07.2004	1940			2300			2480			1820		
09.07.2004	1775			2365			2490			2160		
11.07.2004	1700			2580			2505			1990		
13.07.2004	1675			2260			2785			2010		
14.07.2004	1495	1350	0.90	2775	2510	0.90	2955	2640	0.89	2005	1815	0.91
28.07.2004	1925	1733		2460			2630			2770		
30.07.2004				2630			2600	2323		2790	2526	

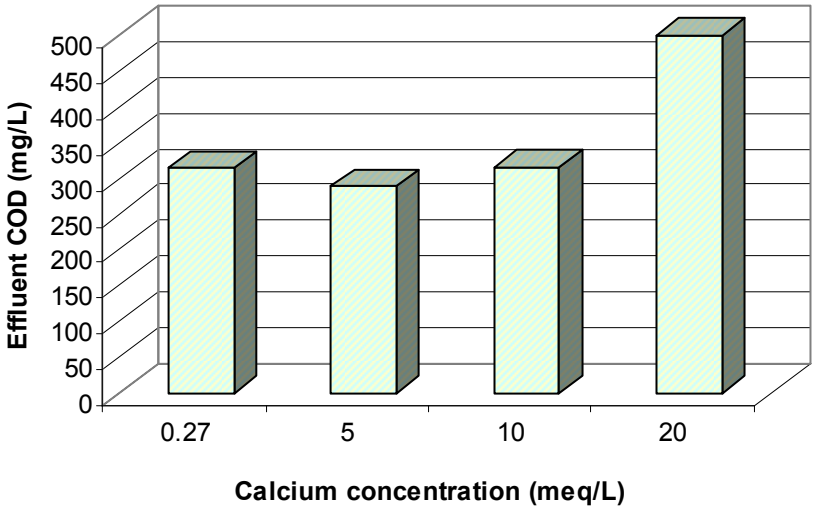


Figure 4.4. Variation of COD removal with Ca concentration

The influent COD to the reactors was 1450 mg/L. Therefore the reactors were operating at a minimum of 65.5% (Ca=20 meq/L) and a maximum of 80.1% COD removal (Ca=5 meq/L). The COD removal efficiencies for reactors having 0.27 meq/L Ca and 10 meq/L Ca are also found to be 78.3% and 78.2% respectively. COD values of the effluents were measured with centrifuging the effluents. As it is going to be demonstrated in the following section, extracellular polymer concentration in the sludge samples increased with increasing calcium concentration. Therefore, the sharp effluent COD increase at 20 meq/L of calcium can be attributed to the considerable amount of polymers at this concentration since soluble EPS also increased proportional to the bound EPS and contributed to the COD of the effluent.

4.2.2. Chemical Characteristics of Activated Sludge With Respect to Calcium Ion Concentration

Extracellular polymeric substances (EPS) constitute the major components of the activated sludge floc matrix and are shown to be important in bioflocculation. Previous researches revealed that EPS is composed of mainly proteins, carbohydarates, nucleic acids, lipids and humic substances (Friedman *et al*., 1969; Pavoni *et al*., 1972; Horan and Eccles, 1986; Urbain *et al*., 1993; Jorand *et al*., 1995; Bura *et al*., 1998; Goodwin and Forster, 1985; Dewalle and Chian, 1974; Frolund *et*

al., 1996). Furthermore it is widely accepted that the relative amounts of EPS constituents is of more significance than their total amount for bioflocculation.

In the following sections the effect of calcium ion concentration on the chemical structure of extracted EPS is discussed in terms of the two major components of EPS which are carbohydrate and protein. In addition, the amount of calcium ion concentration incorporated into the floc structure is also presented.

4.2.2.1. Variations on the production and composition of EPS

As it is mentioned in the previous sections, Cation Exchange Resin (CER) method was applied to extract EPS. This method uses the principle of removal of cations from EPS structure by breaking the metal ion bridges and causes the polymers to hydrolyze from the floc structure and pass into the solution (Urbain *et al*., 1993; Higgins and Novak, 1997a,b; Dignac *et al*., 1998; Sanin and Vesilind, 2000).

The change in the amount of carbohydrate and protein components of EPS as well as the total EPS in relation to calcium ion concentration of the feed are all summarized in Table 4.5. Results show that the quality and quantity of EPS changed significantly when the reactors were operated under different calcium ion concentrations. First of all total amount of EPS increased sharply as the calcium concentration is increased. Examining Table 4.5, total polymer concentration is seen to increase from about 6 mg/gVSS in control reactor to 16, and then to 29 and finally to 40 mg/gVSS for 5, 10 and 20 meq/L of calcium concentrations respectively. It is evident from the data that amounts of both proteins and carbohydrates increased with addition of calcium to the system.

Apart from the total quantity of EPS, one striking finding of this study is that the relative amounts of constituents of EPS changed as the concentration of calcium in the reactors changed. As it is seen from Table 4.5, while protein concentration of EPS was greater in control reactor compared to the carbohydrates, this situation reversed with the addition of calcium ion into the growth medium. The carbohydrates

increased significantly with increasing calcium dose, whereas, proteins did not show that degree of sharp increase.

Table 4.5. Effect of calcium concentration on the production and composition of EPS

Ca Conc. (meq/L)	0.27	5	10	20
Total EPS (mg/gVSS)	6.4	15.8	28.9	40.2
Carbohydrate (mg/gVSS)	1.4	9.7	19.1	25.7
Protein (mg/gVSS)	5.0	6.1	9.8	14.5
Ratio of carbohydrate to protein	0.3	1.6	2.0	1.8

In the literature, contradictory results have been proposed regarding the relative concentration of carbohydrates and proteins in EPS. The predominance of proteins emphasized by Tenney and Verhoff**,** (1973); Brown and Lester, (1980); Barber and Veenstra, (1986); Eriksson and Alm, (1991); Urbain *et al*., (1993); Higgins *et al*., (1997a,b,c); Jorand *et al*., (1998) and Bura *et al*., (1998). However greater amounts of carbohydrates than poteins were reported by Friedman *et al*., (1969); Pavoni *et al*., (1972); Norberg and Enfors, (1982); Goodwin and Forster, (1985), Horan and Eccles (1986); Morgan *et al*., (1990); Bejar *et al*., (1998).

On the other hand, it has been concluded that there can not be a generalization about whether proteins or carbohydrates are dominant constituents of EPS since production of EPS depends on many factors. Studies have shown that growth phase of microorganisms, sludge residence time, C/N ratio of the wastewater, type of the activated sludge and microorganisms, presence of cations, etc. are all affect the production of EPS. This is also the case in this research that depending on the availability and the binding abilities of the divalent cations to the negatively charged groups of EPS (Busch and Stumn, 1968; Pavoni *et al*., 1972; Forster and Lewin, 1972; Bruus *et al*., 1992; Murthy *et al*., 1998; Murthy and Novak, 1999; Higgins and Novak, 1997a,b,c), the total EPS extracted increases as expected. However, most of the studies in the literature used batch experiments and the role of cations on

bioflocculation and sludge properties when they are introduced with the growth media has not been studied thoroughly. Even the few studies that investigated the role of cations in growth media reported conflicting results since they have used different monoculture bacteria which did not represent the wide variety of microorganisms in activated sludge systems. (Tezuka, 1969; Endo *et al*., 1976; Shimizu and Odawara, 1985).

In the study performed by Higgins and Novak (1997a,b), the concentrations of divalent cations were increased in the feed using equimolar concentrations of calcium and magnesium. The increase in divalent cations resulted in an increase in the bound protein concentration, but had little effect on bound polysaccharide concentration. This seems contradictory to the results obtained in our research since it was found that calcium ions caused an increase in polysaccharide concentration. However, in their study, Higgins and Novak (1997a,b) noted that the feed to the laboratory reactors was bactopeptone which is mainly a proteinaceous material (feed COD/N ratio was about 6) and thus it could select for organisms that produce extracellular proteins resulting in differences in biopolymer content. With our system having a COD/N ratio of 17, a value better representing typical activated sludge operation, the differences between our findings and findings of Higgins and Novak (1997a,b) can be explained. Furthermore, they have also reported that although calcium ions were negatively correlated with soluble polysaccharides, interestingly the slime protein and slime polysaccharides were positively correlated indicating that their binding and release mechanisms may be similar.

In our study, which is the case that both glucose and peptone together with the calcium ions were provided in the feed media to the mixed culture microorganisms, it is observed that concentration of polysaccharides in the floc matrix predominates the proteins. Also the ratio of carbohydrates to proteins increases (Table 4.5) when calcium concentration is increased to 10 meq/L. Although this ratio slightly decreases when calcium is further increased to 20 meq/L from 10 meq/L, carbohydrate concentration is higher than proteins for all of the studied calcium concentrations (5, 10 and 20 meq/L).

Besides bridging negatively charges on EPS, the considerable increase in the amount of carbohydrates in the floc network is probably due to calcium induced aggregation/gellation of polysaccharides; an example to such a situation was reported by Bruus *et al*. (1992) and Sanin and Vesilind (1996) for alginate. Alginate is a polysaccharide and its unique composition results in the formation of alginate gels in the presence of calcium ions. Thus, based on the previous research and findings of this study, it can be concluded that calcium ion binds both proteins and carbohydrates in the floc matrix by charge bridging mechanism. However, it has higher affinity to carbohydrates than proteins. Another possible explanation of finding higher EPS carbohydrates at higher calcium concentrations can be that the presence of high calcium concentration in the feed medium induces the production of carbohydrate type polymers by microorganisms.

4.2.2.2. Variations of Calcium Concentrations in Floc Structure

The calcium ions incorporated into the floc matrix was measured. The sludge samples were extracted by microwave assisted digestion method and the results are demonstrated in Figure 4.5.

When Figure 4.5 is examined it is seen that the general trend for the amount of calcium ion concentration incorporated into the floc structure is in increasing direction although a decrease occurred for 10 meq/L reactor. A significant reason of this result could not be identified because according to the EPS amounts extracted using CER technique (Table 4.5) the concentration of calcium in reactor having 10 meq/L calcium should have been in between the values of reactors having 5 and 20 meq/L calcium. However, as previously mentioned the general trend is that the calcium ions in the floc structure increased as their concentration in the feed increased causing greater amounts of EPS to get bind in the floc matrix. This finding is supported by the CER data which shows that total amount of EPS and both of its measured constituents increased with increasing calcium concentration (Table 4.5).

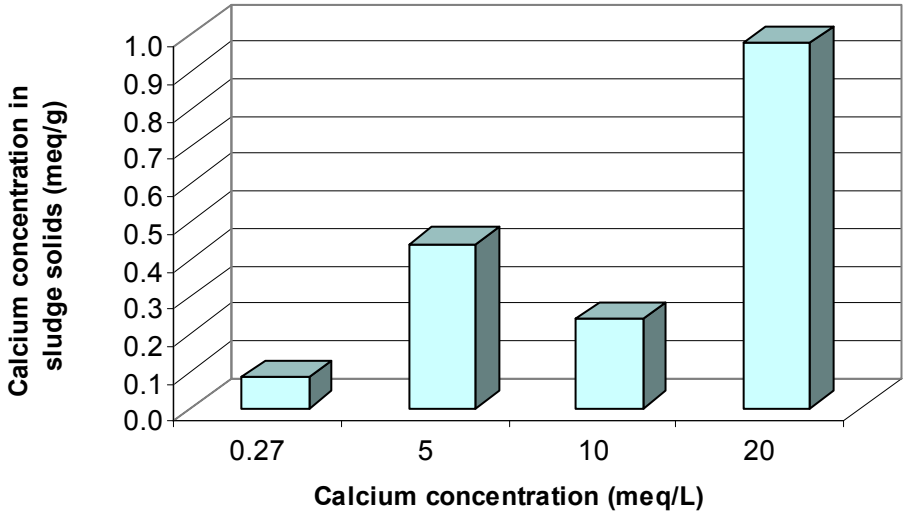


Figure 4.5. Calcium concentration in floc structure with respect to calcium concentration of the feed

4.2.3. Surface Chemical Properties of Activated Sludge with respect to Calcium Ion Concentration

Flocculation is a surface phenomena and thus the surface chemical properties are fundamental for the flocculating ability of activated sludge since they determine the subsequent behavior of sludge in a treatment system such as settleability and dewaterability. In the following sections the results of the experiments regarding the two surface properties of activated sludge, hydrophobicity and zeta potential, are presented and their relations to calcium concentration are discussed.

4.2.3.1. Effects on Hydrophobicity

Hydrophobicity of the sludge samples was determined according to the Microbial Adhesion to Hydrocarbons (MATH) test. It should be emphasized here that this test does not indicate the exact value of hydrophobicity, instead it gives a relative value. The relative hydrophobicities depending on the calcium ion concentration in the reactors are demonstrated in Figure 4.6. As it is seen from the figure, hydrophobicity of the control reactor was 20.2% and it increased to 44.8% and 59.1% for 5 and 10 meq/L feed water calcium concentrations, respectively and then decreased to 49.3%

for 20 meq/L calcium concentration. Depending on the findings demonstrated in Table 4.5 and Figure 4.6, it can be deduced that total amount of EPS including both carbohydrates and proteins increased with increasing calcium concentration of the feed and this resulted in an increase in relative hydrophobicity although not with a smooth trend.

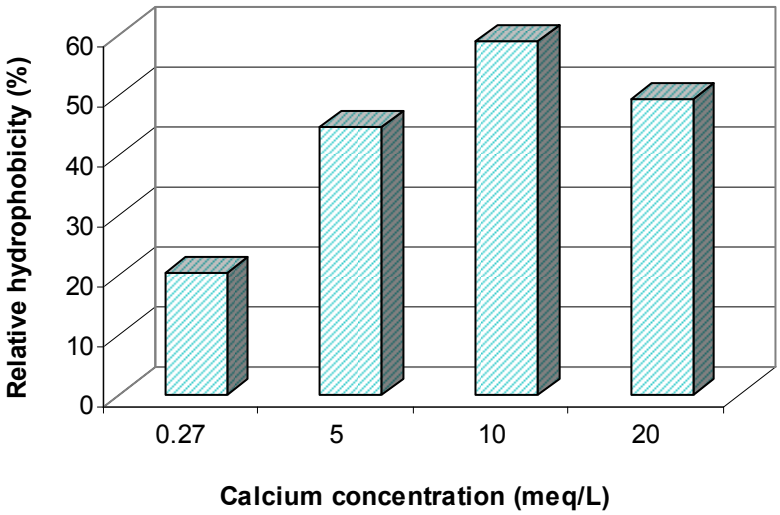


Figure 4.6. Relative hydrophobicity values with respect to calcium concentration

However, in the literature it was reported that the hydrophobic fractions on the cell surface are made up of proteins but not of carbohydrates (Urbain *et al*., 1993; Jorand *et al*., 1998; Liao *et al*., 2001) and mainly amino acids with hydrophobic side chains contribute significantly to the hydrophobicity of microbial surface (Bengtsson, 1991; Dignac *et al*., 1998; Liao *et al*., 2001). Furthermore, it was also stated that the total carbohydrate levels had a negative influence on the hydrophobicity and the presence of a large amount of hydrophilic and mainly neutral carbohydrates might be contributing to the more hydrophilic nature of sludge (Liao *et al*., 2001; Durmaz and Sanin, 2003; Sesay and Sanin, 2004).

In this study, based on the protein concentration data, it is expected that reactor with calcium as 20 meq/L would have the highest hydrophobicity as it contains the highest amount of proteins. However, it was also reported that the balance between the hydrophobic and hydrophilic groups accounts for the overall hydrophobicity

(Daffochio *et al*., 1995). Moreover, Liao *et al*., (2001) proposed that the proportions of EPS components (proteins/carbohydrates and/or proteins/(carbohydrates+DNA)) were more important than the quantities of individual EPS components in controlling hydrophobicity. If the hydrophobicity data is examined from this point of view considering the proportions of carbohydrates and proteins to each other given in Table 4.5 in section 4.2.2.1, it is seen that hydrophobicity is positively correlated to carbohydrate to protein ratio for all of the reactors without any exception.

To be more precise, in contradiction to the aforementioned findings in the literature, this study showed that the relative hydrophobicity of the sludge samples increased with increasing relative amount of carbohydrates compared to the proteins in the presence of excess calcium ions. Similarly, in a recent study, it was also reported that humic substances and protein in EPS showed significant negative correlations to hydrophobicity (Wilen *et al*., 2003a). However, they also observed that more hydrophilic fractions of the polymers were bound to cations.

4.2.3.2. Effects on Surface Charge

It is widely known that all the sludge solids and their extracts carry an overall negative charge (Horan and Eccles, 1986; Sutherland, 1990; Morgan *et al*., 1990; Keiding and Nielsen, 1997). Studies have shown that EPS are anionic in the neutral pH range (Busch and Stumn, 1968; Pavoni *et a*l., 1972; Morgan *et al*., 1990) and contain pyruvate ketals, uronic acids and phosphate groups which also contribute to anionic capacity (Sutherland 1990). In accordance with previous studies, all of the zeta potentials were measured as negative and changed between -22.4 and -38.8 mV as it is shown in Figure 4.7. Similarly, Mikkelsen and Keiding (2002) reported that the zeta potential of activated sludge is in the range of -29.6±8.5 mV which is almost the average value obtained in this research. The negative zeta potential values follow an increasing trend (except for 10 meq/L calcium reactor) with increasing calcium concentration. This result can be attributed to the increasing amount of total EPS as in previous studies during which a positive correlation between total EPS and negative surface charge was observed (Morgan *et al*., 1990; Mikkelsen and Keiding, 2002; Wilen *et al*., 2003a).

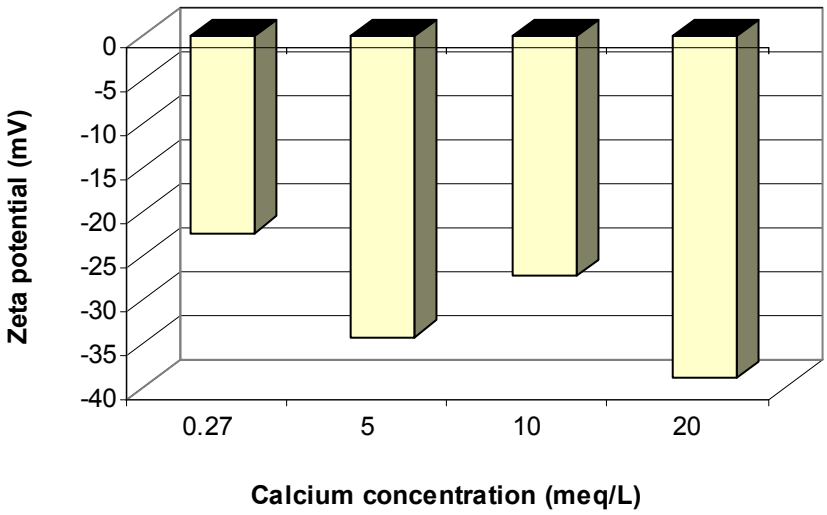


Figure 4.7. Effect of calcium concentration on zeta potential

Even though sludge incorporates calcium ions into the floc matrix to neutralize the negative charges of EPS, it seems that the incorporated calciums are not enough to neutralize the increasing surface charges originating from EPS. In relation with this argument it was also reported that there were negative correlations between the negative surface charge and the amount of mono-, di- and tri-valent cations, because of the fact that cations bound on the floc surface only partially neutralize the negative charged groups (Wilen *et al*., 2003a). However, in our study a positive trend is observed between the negative surface charge and the amount of calcium ions in floc structure. A possible reason of this result is that calcium ions caused considerable amount of increase in EPS especially in the carbohydrates containing mainly negatively charged groups. Some of the functional groups on EPS are neutralized when metal ion binding of polymers occurred, however the other free negative functional groups contribute to the overall charge of EPS.

In addition, Liao *et al*. (2001) suggested that the ratio of proteins to carbohydrates is important in determining the surface charge could be related to the unique charge properties of proteins. The amino groups in proteins carry positive charges, and can neutralize some of the negative charge from carboxyl and phosphate groups and therefore decrease the net negative surface charge of sludge flocs. If the reactor

containing 10 meq/L is excluded the rest of the data in this research supports this statement that protein to carbohydrates ratio decrease representing the higher relative amounts of carbohydrates and this brings about more negative surface charge.

Inverse correlations between hydrophobicity and surface charge have been reported in previous studies (Liao *et al*., 2001; Pere *et al*., 1993). However, in this research, when the general trends are considered a positive correlation is observed between the two parameters. Similarly, based on the weak and moderate correlations between the proportions of EPS components and the hydrophobicity or surface charge Liao *et al*. (2001) also concluded that other factors and/or EPS components might be contributing to the hydrophobic and surface charge properties of microbial floc. A better understanding of hydrophobicity and surface charge requires more fundamental information about the EPS composition and the physical nature of specific EPS molecules (Liao *et al*., 2001).

4.2.4. Physical Properties of Activated Sludge with respect to Calcium Ion Concentration

Separation by settling and the following dewatering step are the key aspects which determine the efficiency and economy of wastewater treatment plants. Sludge viscosity that gives information about how easily the sludge can be pumped is also another physical parameter important for activated sludge systems. The effects of calcium ion concentration on these properties of sludge are investigated and the results are revealed in the foregoing sections.

4.2.4.1. Effects of Calcium on Rheological Properties of Sludge

The rheological properties of sludge samples were determined using a rotational viscometer having a cylindrical spindle (Brookfield, LVDVII+, with ultra low viscosity adapter). For each calcium concentration, shear stress vs. shear rate curve is plotted and its conformity to different sludge flow models including Newtonian, Bingham plastic non-Newtonian and pseudoplastic non-Newtonian which are described in section 2.5.1 are tested. As can be seen from Figure 4.8 the curves fitted

give highest fit to power law equation as in the case of pseudoplastic fluids. The power law of non-Newtonian pseudoplastic flow behavior is described by the following formula:

$$\tau = K (du/dy)^n \quad \text{.....(4.1)}$$

where, τ is shear stress, K is the fluid consistency index (a term analogous to the viscosity term), du/dy = shear rate and n is the flow behavior index. The value of n is smaller than 1 for pseudoplastic fluids and its magnitude shows the amount of deviation from Newtonian flow. The more the deviation of n from 1 means that the more the fluid deviates from Newtonian behavior and the flocs are more breakable.

The model fit parameters to non-Newtonian pseudoplastic flow for the cation concentrations studied are demonstrated in Table 4.6. When the K and n values are examined in Table 4.6, it is clear that as calcium concentration is increased the value of K decreases whereas the value of n increases except for reactor with 20 meq/L calcium. Looking at the K values in Table 4.6 it is seen that K is 1.4975 dyne.cm^{-2}.s at 0.27 meq/L calcium concentration and decreased to 0.6024 dyne.cm^{-2}.s when cation concentration is increased to 20 meq/L.

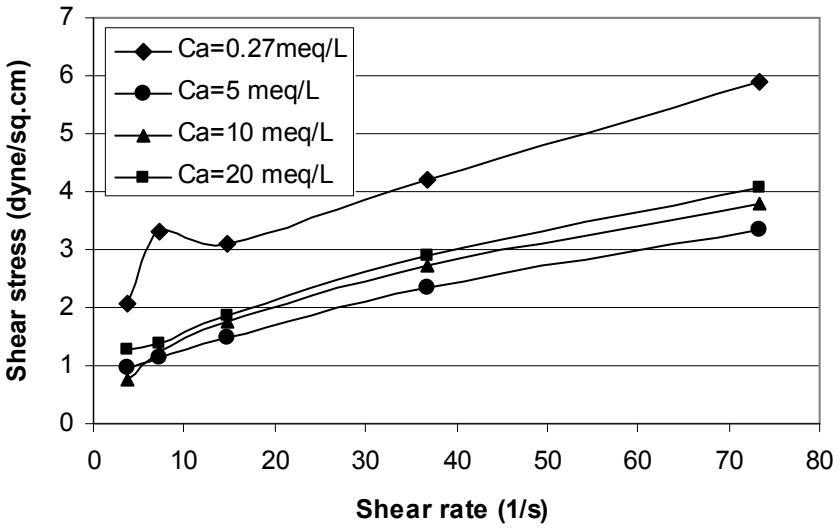


Figure 4.8. Rheogram of activated sludge at different calcium concentrations

The decrease in K values means that sludge becomes less viscous as the calcium ion concentration increases. Examining the n values at different calcium concentrations, it is obvious that n is pretty much the same but it is definitely far away from 1 indicating that in all cases flocs are strongly non-Newtonian.

Table 4.6. Effect of calcium concentration on model fit parameters for non-Newtonian flow

Ca conc. (meq/L)	K	n	R^2	Equation
0.27	1.4975	0.3063	0.9041	$\tau = K (du/dy)^n$
5	0.5159	0.4219	0.9856	
10	0.4152	0.5222	0.9943	
20	0.6024	0.4359	0.9931	

The same trend for rheological properties of sludge samples is also confirmed by the apparent viscosity values which are demonstrated in Figure 4.9. The apparent viscosity is determined at a fixed shear rate and fixed solids concentration and represents a value independent of solids concentration. In order to examine the effect of calcium concentration on the viscosity, apparent viscosities were measured at fixed shear rate of 36.7 s^{-1} and a variety of solids concentrations. Once the viscosity versus solids concentration graphs are plotted as in Figure 4.9, the apparent viscosity is read graphically at solids concentration of 5000 mg/L. From Figure 4.9 it was observed that the dependence of viscosity on solids concentration fits to an exponential law equation. The apparent viscosities were read from the graph as 7.67 ; 3.81 ; 2.47 and 2.49 cP for 0.27 ; 5; 10 and 20 meq/L calcium concentrations, respectively. These results are consistent with the K values in Table 4.6. One striking finding is that very close values are obtained for reactors having 10 and 20 meq/L calcium providing that further increasing of calcium from 10 meq/L to 20 meq/L does not result in an improvement in viscosity and pumpability of the sludge.

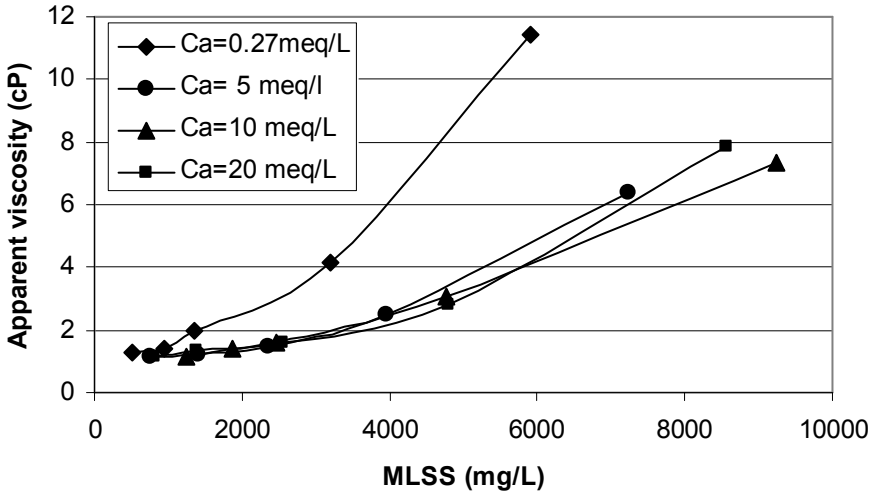


Figure 4.9. Apparent viscosities with respect to calcium concentration at a shear rate of 36.7 s^{-1}.

4.2.4.2. Effects of Calcium on Dewaterability of Sludge

Dewaterability of sludge samples with respect to increasing calcium concentration is determined using Specific Resistance to Filtration (SRF) test and the results are depicted in Figure 4.10.

It is evident from Figure 4.10 that a very sharp decrease occurred in SRF values with the addition of 5 meq/L calcium. As calcium ions increased to 5 meq/L from control reactor level (0.27 meq/L) SRF decreased by about 0.65 logs (Table 4.7). Further increase of calcium ions to 10 meq/L resulted in a mild decrease (0.07 logs) and a very close value to the SRF value at 5 meq/L calcium was measured. However, as calcium concentration was increased further from 10 meq/L to 20 meq/L, SRF decreased by another 0.3 logs. When all of the data are considered SRF values decreased from $3.6*10^{14}$ to $3.80*10^{13}$ m/kg corresponding over 0.9 logs on the whole. According to the previous studies in the literature the SRF values of activated sludge change between 10^{10} and 10^{14} m/kg which is in agreement with the findings of this research.

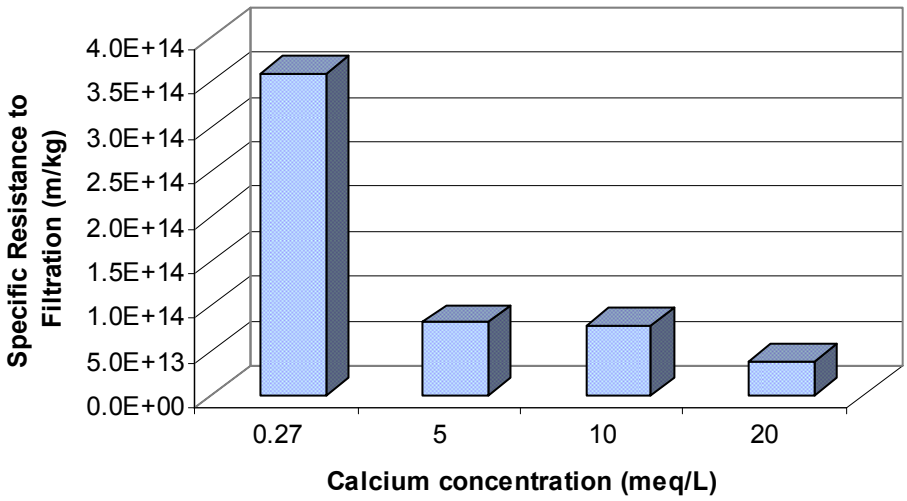


Figure 4.10. Effect of calcium concentration on filterability of activated sludge at 70.91 kPa pressure.

Results of the SRF test highlight that filterability of the sludge increased as total EPS increased depending on the calcium concentration. This finding is also supported by Mikkelsen and Keiding (2002) who observed that with high EPS contents sludges had a lower shear sensitivity and lower degree of dispersion. This in turn led to a better filterability in terms of low resistance to filtration (SRF). Moreover, Jin *et al.* (2004) reported that high amount of the individual and total polymers in the extracted EPS corresponded to a good dewaterability. Jin *et al*. (2004) also observed that the surface properties measured by the relative hydrophobicity and surface charge had significant impacts on dewaterability. High values of relative hydrophobicity and surface charge corresponded to poor dewaterability. On the contrary, in our study opposite relationship was observed between the three parameters such that dewaterability improved with increasing hydrophobicity and negative surface charge. Similar to our results, Durmaz and Sanin (2003) and Sesay and Sanin (2004) also reported that dewaterability improved with increasing hydrophobicity. However, unlike to our results, dewaterability decreased with increasing negative surface charge (Durmaz and Sanin ,2003).

Wu *et al*. (1982) stated that sludge dewaterability is closely related to sludge floc-forming ability. When activated sludge flocs are large and strong, its filtering

property is excellent. The hypothesis of our study indicates that increasing calcium concentration improves floc formation and as expected smallest SRF value is obtained for the highest calcium concentration confirming the above statement. It was also reported by Higgins and Novak (1997a), Keiding and Nielsen (1997) and Jin *et al*. (2004) that high concentrations of divalent and trivalent metal ions were related to low values of bound water and so improved dewaterability.

When the SRF values and effluent supernatant turbidities after 1 hour settlement are compared to each other no correlation is observed between the two parameters. Bruus *et al*. (1992) detected positive correlation between SRF and turbidity and stated that either the small particles or colloids are responsible for filterability decrease. The measured turbidity values presented in Table 4.7 are said to be in conformity with the above statement for 0.27, 5 and 10 meq/L calcium concentrations, however, the highest turbidity value is observed at 20 meq/L concentration which has the smallest SRF value. On the other hand, all the turbidities measured are very low already and are well within the acceptable levels. Therefore, obtaining no correlation with the SRF value is plausible since there were not any significant number of particles to interfere with the SRF measurement.

Table 4.7. Summary of the other physical properties of sludge at different calcium concentrations.

Ca conc. (meq/L)	0.27	5	10	20
SRF (m/kg)	$3.6*10^{14}$	$8.18*10^{13}$	$7.85*10^{13}$	$3.80*10^{13}$
Turbidity (NTU)	19.5	12.5	13	26.5

4.2.4.3. Effects of Calcium on Settling Characteristics of Sludge

Zone settling velocity (ZSV) is one of the ways to determine sludge settleability and it is used as a design parameter to assess how much secondary clarifiers can be loaded. For a specific sludge, the ZSV is a function of suspended solids concentration (Jin *et al*., 2003). For this reason, for each reactor sludge solids concentration versus zone settling velocity graphs are prepared as given in

Appendix C. From these graphs ZSV vs. calcium concentration graph is plotted at a fixed solids concentration of 3500 mg/L MLSS (Figure 4.11) in order to eliminate the effect of concentration.

According to Figure 4.11 the addition of 5 meq/L calcium caused a considerable increase in ZSV. At 0.27 meq/L calcium concentration, the ZSV was $7.4*10^{-4}$ cm/s which was a quite low value and it sharply increased by 71 folds to $53*10^{-3}$ cm/sec with addition of 5 meq/L calcium. When calcium was further increased to 10 and 20 meq/L the velocity slightly decreased to $47*10^{-3}$ and to $40*10^{-3}$ cm/s, respectively. The reason of this decrease might be attributed to the increased resistance to settlement as a result of the increase in the amount of total EPS. The friction with the water molecules is increased by the EPS network decreasing the zone settling velocity. But this decrease is considered not as a problematic decrease since it is very small.

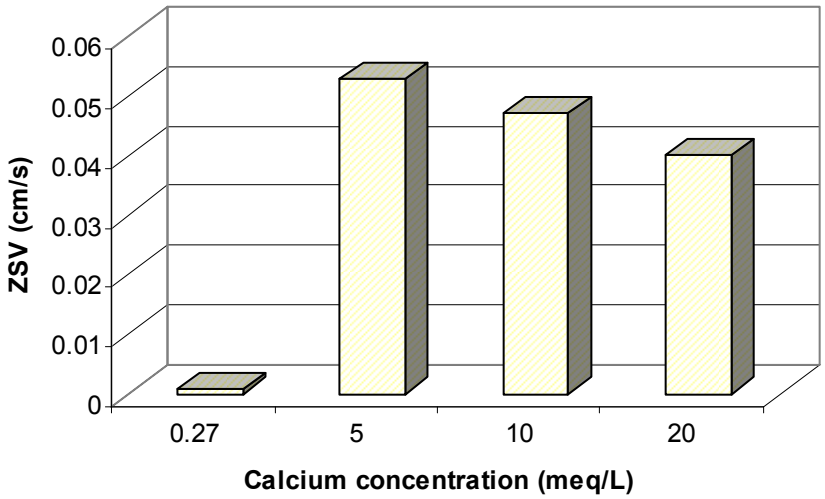


Figure 4.11. Zone settling velocities at different calcium concentrations at a fixed MLSS concentration of 3500 mg/L

Sludge volume index (SVI) is another key parameter to define sludge settleability. SVI values values less than 150 indicate well settling sludge and if this value is exceeded settling problems occur such as bulking (Goodwin and Forster, 1985). As it is demonstrated in Figure 4.12, SVI value decreased significantly when the calcium

concentration was increased to 5 meq/L from control reactor levels. For control reactor SVI was 213 mL/g indicating a bulking situation and addition of 5 meq/L calcium decreased SVI to 72 mL/g and cured sludge bulking completely. Further increase of calcium ion to 10 meq/L resulted in a small increase in SVI value causing it to increase to 85 and it stayed almost in the same level when calcium is continued to increase to 20 meq/L. All of the results obtained at 5, 10 and 20 meq/L calcium indicate that there are no settlement problems experienced in these sludges. When the trends observed in ZSV and SVI values are examined it is evident that there is a good correlation between them for all of the measured calcium concentrations.

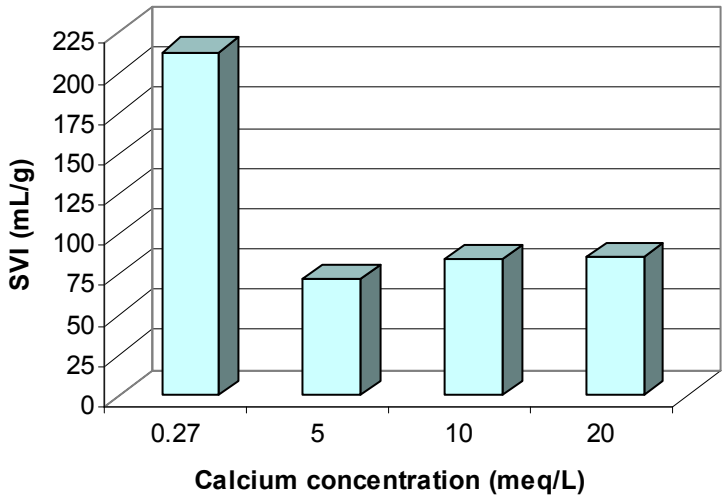


Figure 4.12. SVI values at different calcium concentrations

It can be concluded that calcium concentrations greater than 5 meq/L resulted the settling characteristics to deteriorate slightly which can be attributed to the greater amounts of total polymers. However, the SVI values at all calcium concentrations were smaller than 150 indicating good settleability. On the other hand, Biggs *et al*. (2001) reported that for concentrations of less than 8 meq/L of calcium no significant increase in floc size was observed even though an increase in the initial rate of change of floc size was seen. Addition of calcium greater than 8 meq/L resulted in a dramatic increase in floc size and settleability.

Moreover, an increase in divalent cation concentration (Ca and Mg) in the feed to the reactors was associated with an increase in the bound exocellular protein concentration and this also resulted in an improvement in settling and clarification (Higgins *et al*. 1997 b,c). Jin *et al*. (2003) also reported that cations like Ca, Mg, Al and Fe in the sludge improved the sludge compressibility and settleability significantly. However, they have observed that the quantity of the polymeric compounds, protein, humic substances and carbohydrate in the sludge and the extracted EPS had significant positive correlations with SVI, whereas ZSV was independent of the polymeric constituents of the sludge. Sludge containing high concentration of the extracted EPS was concluded to have poor compressibility and settleability (Jin *et al*., 2003).

Sponza (2004) observed that the content of protein and total EPS showed a strong inverse linear correlation with SVI as with Goodwin and Forster, 1885; Yun *et al*., 2000 who also showed that SVI decreased with increasing EPS. However some other researchers observed no correlation between SVI and EPS (Chao and Keinath, 1979; Jorand *et al*., 1998; Liao *et al*., 2001). Similarly, Sponza (2004) and Andreadakis (1993) also could not observe a correlation between the total carbohydrate content and the SVI.

From the results of the physical analyses it is seen that rheological characteristics, dewaterability and settleability of sludge improved significantly at the calcium ion concentration of 5 meq/L. Above this value, settleability and rheological characteristics did not get better and even slightly deteriorated whereas dewaterability continued to improve. However, the degree of improvement in dewaterability was not as sharp as it was at 5 meq/L when shifted to 10 and 20 meq/L. Hence, 5 meq/L of calcium concentration can be regarded as the optimum dose for proper operation activated sludge systems. If dewaterability is the first concern for an activated sludge system then calcium concentrations greater than 5 meq/L are also suggested.

CHAPTER 5

CONCLUSIONS

In this research the effects of calcium ion concentration was investigated first on sludge bulking condition in a phosphorus deficient medium and second on physical, chemical and surface properties of a non-deficient sludge in order to improve the settleability, dewaterability, and pumpability of activated sludge.

The specific results obtained from the study are presented as follows:

- Phosphorus deficiency in the feed medium of the reactors resulted in a serious sludge bulking problem. SVI values were excessively high. The microphotographs of the sludge samples revealed that sludges from all of the reactors independent from calcium concentration exhibited viscous bulking with no obvious filamentous microorganisms present.

- Analysis of sludge indicated that the sludges were rich in terms of carbohydrate content under strictly phosphorus limited condition. The high carbohydrate was thought to originate from the possible stimulating effect of calcium ions on the production of EPS.

- The settling characteristics of the sludge samples improved as the phosphorus concentration in the feed was increased to half of the stoichiometrically required value (COD/N/P = 100/5/0.5). SVI values of all of the three sludges fell within the acceptable range.

- Parallel to the settlement results, microscopic examinations showed that the floc structure for all the reactors changed significantly. Some filamentous microorganisms showed up in the flocs indicating a healthier floc formation and the viscous bulking problem disappeared to a great extent.

- Microscopic investigations have shown some granular structures in the microphotographs after the amount of phosphorus in the feed was increased. Microorganisms starved for phosphorus, seemed to accumulate polyphosphate granules when they were exposed to a phosphorus source.

- The reactors operated under phosphorus limited situation had very low MLSS values. When the same reactors were operated at non-limiting phosphorus concentration they were able to produce MLSS values around 2500-3000 mg/l at steady state.

- Physical, chemical and surface-chemical properties of activated sludge changed significantly in relation to calcium ion concentration when reactors were operated under non-limiting phosphorus condition.

- One of the most important effects of calcium was observed in the production of EPS. Total EPS (both carbohydrates and proteins) increased with increasing calcium dose. However, carbohydrate content of EPS increased in greater amounts than protein and carbohydrates dominated proteins at all studied calcium concentrations except for the control reactor.

- Hydrophobicity and surface charge increased (although not with a smooth trend) with calcium concentration depending on the increase in carbohydrate and protein contents of EPS.

- Rheological properties of sludge were positively affected by calcium addition. Viscosity decreased considerably at 5 meq/L calcium concentration and continued to decrease with a smaller amount at 10 meq/L indicating better pumpability. Above 10 meq/L, no further improvement was observed.

- Settleability improved significantly at 5 meq/L dose of calcium as indicated by a sharp increase in ZSV and sharp decrease in SVI. On the other hand, settleability did not change any further with increasing calcium dose to 10 and 20 meq/L.

- Filterability of sludge improved considerably when calcium concentration shifted to 5 meq/L from control reactor levels which represented better dewaterability and continued to improve at 10 and 20 meq/L calcium concentrations also, however, with smaller amounts.

- Based on the results obtained, it is concluded that calcium ions increased the degree of bioflocculation causing stronger flocs to form. One of the reasons of calcium induced bioflocculation is attributed to the compression of the negatively charged double layer around the microorganisms by calcium ions which facilitated the microorganisms to get closer to each other. Furthermore, a stimulating effect of calcium on the production of EPS is proposed. Formation of stronger flocs with the higher quantity of EPS production is shown to be due to the bridging ability of calcium ions of the negatively charged groups of EPS. Subsequently, the improvements in bioflocculation resulted in better settling and dewatering properties of sludge and according to the experimental results, 5 meq/L of calcium concentration in the influent is suggested to be the optimum dose for activated sludge systems.

CHAPTER 6

RECOMMENDATIONS FOR FUTURE STUDY

This research revealed that calcium ion present in the feed resulted in viscous bulking independent from the studied concentrations when phosphorus was deficient in the medium. In a further study, phosphorus deficient condition can be investigated with other types of cations in order to determine their effects on the type of bulking and other sludge characteristics.

In this study, it has been shown that under non-limiting phosphorus condition, physical properties of activated sludge improved significantly when calcium concentration is shifted from 0.27 meq/L to 5 meq/L. The concentrations between these two values can be studied to detect the exact point in which the improvements begin to occur.

Finally, a future research on calcium concentrations greater than 20 meq/L can also be conducted in order to find out whether there is a limit concentration above which the amount extracellular polymers does not change and dewaterability does not improve further.

REFERENCES

1. Adamson A. W. Physical chemistry of surfaces, 5th ed. NY, USA: Wiley, 1990.

2. Allison D. G. and Sutherland I. W: (1987). The role of exopolysaccharides in adhesion of freshwater bacteria. *J. Gen. Microbiol*., **133**, 1319-1327.

3. Allison D. G., Evans D. J., Brown M. R. W. and ilbert P. (1990). Possible involvement of the division cycle in dispersal Escherichia coli from biofilms. *J. Bacteriol*., **172**(3), 1667-1669.

4. Andreadakis D. A. (1993). Physical and chemical properties of activated sludge. *Wat. Res*., **27**(12), 1707-1714.

5. APHA (1989). *Standard Methods for the Examination of Water and Wastewater*. 20th Edition. American Public Health Association, American Water Work Association, Water Environment Federation, Washington, D.C.

6. Ault-Riche, D., Fraley, C.D., Tzeng, C.M., and Kornberg, A. (1998). Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in Escherichia coli. *J. Bacteriol*., **180**, 1841-1847.

7. Azeredo J., Vissser J. and Oliveria R. (1999). Exopolymers in bacterial adhesion: interpretation in terms of DLVO and XDLVO theories. *Colloids and Surfaces,* **14**, 141-148.

8. Barahona L. and Eckenfelder W. W. (1984). Relationships between organic loading and zone settling velocity in the activated sludge process. *Wat. Res*., **18**(1), 91-94.

9. Barber J. B. and Veenstra J. N. (1986). Evaluation of biological sludge properties influencing volume reductions. *J. Water Pollut. Control Fed.*, **58**, 149-156.

10. Behn V. C. (1962). Experimental determination of sludge floe parameters. *J. Sanitary Eng. Div, Proceedings ASCE*, **88**(SA3), 39-54.

11. Bejar V., Llamas I., Calvo C. and Quesada E. (1998). Characterization of exopolysaccharides produced by 19 halophilic strains of the species *Halomanas eurihalina*. *J. Biotech.*, **61**, 135-141

12. Bender, J., Rodriguez-Eaton, S., Ekanemesang U.M. and Phillips P. (1994). Characterization of metal-binding bioflocculants produced by the cyanobacterial component of mixed microbial mats. *Appl. Env. Microbiol.*, **60,** 2311.

13. Bengtsson G. (1991). Bacterial exopolymer and PHB production in fluctuating ground-water habitats. *FEMS Microbiol Ecol*., **86**, 15-24.

14. Biggs C. A., Ford A. M. and Lant P. A. (2001). Activated sludge flocculation: direct determination of calcium ions. *Wat. Sci. Tech*., **43**(11), 75-80.

15. Bisogni J. J. and Lawrence A. W. (1971). Relationships between biological solids retention time and settling characteristics of activated sludge. *Water Res*., **5**, 753-763.

16. Bitton G., Wastewater Microbiology. NY, USA: Wilet-Liss, Inc., 478 pages, 1994.

17. Bonting C. F. C., Kortstee G. J. J., Boekestein A. and Zehnder A. J. B. (1993). The elemental composition dynamics of large polyphosphate granules in *Acinetobacter* Strain 210A. *Arch. Microbiol*., **159**, 428-434.

18. Bowen P. T. and Keinath T. M. (1984). Sludge conditioning: effects of sludge biochemical composition. *Wat. Sci. Tech*., **17**, 505-515.

19. Boyette S. M., Lovett J. M., W. G. and Soares J. A. (2001). Cell surface and exopolymer characterization of laboratory stabilized activated sludge from a beverage bottling plant. *Wat. Sci. Tech*., **43**(6), 175-184.

20. Brown M. J. and Lester J. N. (1980). Comparison of bacterial extracellular polymer extraction methods. *Appl. Envir. Microbiol*., **40**, 179-185.

21. Bruus J. H., Nielsen P. H. and Keiding K. (1992). On the stability of activated sludge flocs with implications to dewatering. *Wat. Res*., **26**(12), 1597-1604.

22. Buchan L. (1981). The location and nature of accumulated phosphorus in seven sludges from activated sludge plants which exhibited enhanced phosphorus removal. *Water SA,***7**, 1-7.

23. Bura R., Cheung M., Liao B., Finlayson J., Lee B. C., Droppo I. G., Leppard G. G. and Liss S. N. (1998). Composition of extracelular polymeric substances in the activated sludge floc matrix. *Wat. Sci. Tech*., **37**(4-5), 325-333.

24. Burdman S., Jurkevitch E., Diaz E. M., Serrano A. M. G. and Okon Y. (2000). Extracellular polysaccharide composition of *Azospirillum brasilense* its relation with cell aggregation. *FEMS Microbiol. Letters*, **189**, 259-264.

25. Busch P. and Stumn W. (1968). Chemical interactions in the aggregation of bacteria, bioflocculation in waste treatment. *Environ. Sci. Technol.* **2**(1), 49-53.

26. Butterfield C. T. (1935). Studies of sewage purification. II. A *Zooglea*-forming bacterium isolated from activated sludge. *Pub. Health. Rept*., **50,** 671.

27. Chao A. C. and Keinath T. M. (1979). Influence of process loading intensity on sludge clarification and thickening characteristics. *Wat. Res*., **13**, 1213-1223.

28. Clauss F., Balavoine C., Helaine D. and Martin G. (1999). Controlling the settling of activated sludge in pulp and paper wastewater treatment plants. *Wat. Sci. Tech*., **40**(11-12), 223-229.

29. Curds C. R., Cockburn A. and Vandyke J. M. (1968). An experimental study of the role of the ciliated protozoa in the activated sludge process. *Water Polln. Control*, **67**, 312.

30. Curds C. R. and Fey G. J. (1969). The effect of ciliated protozoa on the fate of *Esherichia coli* in the activated sludge process. *Water Res*., **3**(11), 853-867.

31. Curds C. R. (1975). The role of protozoa in the activated sludge process. *Amer. Zool*., **13**, 161.

32. Daffonchio D., Thaveesri J. and Verstraete W. (1995). Contact angle measurement and cell hydrophobicity of granular sludge from upflow anaerobic sludge bed reactors. *Appl. Environ. Microbiol.*, **61**(10), 3676-3680.

33. Davies D. G. and Geesey G. G. (1995). Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. *Appl. Environ. Microbiol*., **61**, 860– 867.

34. DeCampo R., Souza W., Miranda K., Rohloff P., and Moreno S.N.J. (2005). Acidocalcisomes – Conserved from Bacteria to Man, *Nature Review/Microbiology*, 3, 251-261.

35. Dentel S. K. (1997). Evaluation and role of rheological properties in sludge management. *Wat. Sci. Tech*., **36**(11), 1-8.

36. Dewalle F. B. and Chian S. K. (1974). Kinetics of formation of humic substances in activated sludge systems and their effect on flocculation. *Biotechnol. Bioeng.,* **16**, 739-755.

37. Dick R. I. and Ewing B. B. (1967).The rheology of activated sludge. *J. Water Pollut. Control Fed.,* **39**(4), 543-560.

38. Dick R. and Vesilind P. A. (1969). The sludge volume index – What is it? *J. Water Pollut. Control Fed.*, **41**(7), 1285-1291.

39. Dignac M. F., Urbain V., Rybacki D., Bruchet A., Snidaro D. and Scribe P. (1998). Chemical description of extracelular polymers: Implication on activated sludge floc structure. *Wat. Sci. Tech*., **38**(8-9), 45-53.

40. Dubois M., Gilles K. A., Hamilton J. K., Rebers P. A. and Smith F. (1956). Colorimetric method for the determination of sugar and related substances. *Anal. Chem.,* **28**, 350-356.

41. Durmaz B. (2001). Effect of carbon to nitrogen ratio on the composition of microbial extracellular polymers in activated sludge. M. Sc. Dissertation, Dept. of Environmental Eng. Middle East Technical University, Turkey.

42. Durmaz B. and Sanin F. D. (2001). Effect of carbon to nitrogen ratio on the composition of microbial extracellular polymers in activated sludge. *Wat. Sci. Rech.*, 44(1), 221-229.

43. Durmaz B. and Sanin F. D. (2003). Effect of carbon to nitrogen ratio on the physical and chemical properties of activated sludge. *Env. Tech*., **24**, 1331-1340.

44. Eikelboom D. H. (1975). Filamentous organisms observed in activated sludge. *Water Res*., **9**, 365-388.

45. Eikelboom D. H. and van Buijsen H. J. J. (1981). *Microscopic Sludge Investigation Manual*. TNO Res. Inst. For Env. Hygiene, Delft, The Netherlands.

46. Endo T., Nakamura K. and Takahashi J. (1976). Pronase-susceptible floc forming bacteria: Relationship between flocculation and calcium ion. *Agric. Biol. Chem*., **40**, 2289.

47. Eriksson L. (1987). Conditioning biological sludges with cationic polyelectrolytes. *Wat. Sci. Tech*., **19**, 859-868.

48. Eriksson L. and Alm B. (1991). Study of flocculation mechanisms by observing effects of a complexing agent on activated sludge properties. (1991). *Wat. Sci. Tech*., **24**, 21-28.

49. Ericsson B. and Eriksson L. (1988). Activated sludge characteristics in a phosphorus depleted environment. *Water Res.,* **22**(2), 151-162.

50. Figueroa L. A. and Silverstein J. A. (1989). Ruthenium redadsorption method for measurement of extracellular polysaccharides in sludge flocs. *Biotechnol. Bioeng*., **33**, 941-947.

51. Finlayson J. C., Liao B., Droppo I. G., Leppard G. G. and Liss S. N. (1998). The relationship between the structure of activated sludge flocs and the sorption of hydrophobic pollutants. *Wat. Sci. Tech*., **37** (4-5), 353- 357.

52. Forster C. F. (1968). The surface of activated sludge particles in relation to their settling characteristics. *Water Res*., **2**(11), 767-776.

53. Forster C. F. (1971). Activated sludge surfaces in relation to the sludge volume index. *Water Res*., **5**, 861-870.

54. Forster C. F. and Lewin D. C. (1972). Polymer interactions at activated sludge surfaces. *Effl. Wat. Treatment J*., **12**, 520.

55. Forster C. F. and Dallas-Newton J. (1980). Activated sludge settlement-some suppositions and suggestions. *Water Pollution Control*., **79**, 338-351.

56. Forster C. F. (1982). Sludge surfaces and their relationship to the rheology of sewage sludge suspensions. *J. Chem. Tech. Biotech*.,**32**, 799-807.

57 Forster C. F. (1983). Bound water in sewage sludges and its relationship to sludge surface and sludge viscosities. *J. Chem. Technol. Biotechnol*., **33B**, 76-84.

58. Forster C. F. and Clarke A. R. (1983). The production of polymer from activated sludge by ethanplic extraction and its relation to treatment plant operation. *Water Pollut. Cont.*, **82**, 430-433.

59. Forster C. F. (1985a). Factors involved in the settlement of activated sludge-I Nutrients and surface polymers. *Wat. Res*., **19**(10), 1259-1264.

60. Forster C. F. (1985b). Factors involved in the settlement of activated sludge-II The binding of polyvalent metals. *Wat. Res*., **19**(10), 1265-1271.

61. Forster C. F. (2002). The rheological and physico-chemical characteristics of sewage sludges. *Enzyme and Microbial Technology*, **30**, 340-345.

62. Friedman B. A. *et al*. (1969). Structure of exocellular polymers and their relationship to bacteria flocculation. *J. Water Pollut. Control Fed.*, **98**, 1328-1334.

63. Frolund B., Palmgren R., Keiding K. and Nielsen P. H. (1996). Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Res*., **30**, 1749-1758.

64. Frolund B., Keiding K. and Nielsen P. (1994). A comparative study of biopolymers from a conventional and advanced activated sludge treatment plant. *Water Sci. Tech*., **29**(7), 137-141.

65. Ganczarczyk J. J., Activated Sludge Process: Theory and Practice. USA: Marcel Dekker, Inc., 266 pages, 1983.

66. Gaudy, A and Gaudy, E. Microbiology for environmental scientists and engineers, NY, USA: McGraw Hill, 736 pages, 1980.

67. Gaudy A. F. Jr., Yang P. Y. and Obayashi A. W. (1971). Studies on the total oxidation of activated sludge with and without hydrolytic pretreatment. (1971). *J. Water Pollut. Control Fed.*, **43**, 40-54.

68. Gehr R. and Henry J. G. (1983). Removal of extracellular material, techniques and pitfalls. *Water Res*., **17**(12), 1743-1748.

69. Gerardi M. H. (chair). Wastewater Biology: The Life processes. *Prepared by* Task Force on Wastewater Biology: The Life Processes, USA: Water Environment Federation, 180 pages, 1994.

70. Goodwin J. A. S. and Forster C. F. (1985). A further examination into the composition of activated sludge surfaces in relation to their settlement characteristics. *Wat. Res*., **19**(4), 527-533.

71. Grady C. P. L., Kirsch E. J., Koczwara M. K., Trgovcich B. and Watt R. D. 1984. Molecular weight distributions in activated sludge effluents. *Water Res*., **18**, 239-246.

72. Gregory J. (1989). Fundamentals of flocculation. *CRC Critical Rev. Environ. Control*., **19**, 185-209.

73. Gulas V., Bond M. and Benefield L. (1979). Use of extracellular polymers for thickening and dewatering activated sludge. *J. Water Pollut. Control Fed.,* **51**(4), 798-807.

74. Hale F. D. and Garver S. R. (1983). 'Viscous Bulking of Activated Sludge,' presented at the 56th Ann. Conf. Water Polln Control Fedn, Atlanta, GA, Oct. 2-7, 1983.

75. Harris R. H. and Mitchell R. (1973). The role of polymers in microbial aggregation. *A. Rev. Microbiol*., **27**, 27-50.

76. Hawkes H. A., The Ecology of Wastewater Treatment. NY, USA: Macmillan, 1963.

77. Healy T. W. and Lamer V. K. (1962). Adsorption-flocculation reactions of a polymer with an aqueous colloidal dispersion. *Jour. Physical Chem*., **66**, 1835.

78. Heimenz P. C. Principles of Colloid and Surface Chemistry. 2nd ed. NY: Marcel Dekker, Inc., 1986.

79. Hejzlar J. and Chudoba J. (1986). Microbial polymers in the aquatic environment-I. Production by activated sludge microorganisms under different conditions. *Water Res*., **20**(10), 1209-1216.

80. Heukelekian H. and Littman M. L. (1939). Carbon and nitrogen transformations in the purification of sewage by the activated sludge process. II. Morphological and biochemical studies of *Zoogleal* organisims. *Sew. Works Jour*., **11**, 752.

81. Higgins I. J. and Burns R. G., The Chemistry and Microbiology of Pollution, London, England: Academic, 1975.

82. Higgins M. J. and Novak J. T. (1997a). The effect of cations on the settling and dewatering of activated sludge: Laboratory results. *Water Environ. Res*., **69,** 215-224.

83. Higgins M. J. and Novak J. T. (1997b). Dewatering and settling of activated sludges: The case for using cation analysis. *Water Environ. Res*., **69,** 225-232.

84. Higgins M. J. and Novak J. T. (1997c). Characterization of exocellular protein and its role in bioflocculation. *J. Env. Eng.,* **123**(5), 479-485.

85. Higgins M. J., Tom L. A. and Sobeck D. C. (2004). Case study I: Application of the divalent cation bridging theory to improve biofloc properties and industrial activated sludge system performance-direct addition of divalent cations. *Water Environ. Res*., **76(**4), 344-352.

86. Hoa P. T., Nair L. and Visvanathan C. (2003). The effect of nutrients on extracellular polymeric substance production and its influence on sludge properties. *Water SA.,* **29**(4), 437-442.

87. Horan N. J. and Eccles C. R. (1986). Purification and characterization of extracellular polysaccharide from activated sludges. *Wat. Res*., **20**(11), 1427-1432J.

88. Horan N. J., Biological Wastewater Treatment Systems: Theory and Operation. England: John Wiley & Sons, Inc., 307 pages, 1990.

89. Houghton J. I., Quarmby J. And Stephenson T. (2001). Municipal wastewater sludge dewaterability and the presence of microbial extracellular polymer. *Wat. Sci. Tech*., **44**(2/3), 373-379.

90. Hsieh K.M., Murgel G. A., Lion L. W. and Shuler M. L. (1994). Interactions of microbial bio.lms with toxic trace metals 1. Observation and modeling of cell growth, attachment, and production of extracellular polymer. *Biotechnol Bioeng*., **44**, 219–31.

91. Jahn A. and Nielsen P. H. (1996). Extraction of extracellular polymeric substances from biofilms using a cation exchange resin. *Water Sci. Tech*., **32**(8), 157-164.

92. Jahn, A., Nielsen, P. H. and Palmgren, R. (1997). Exopolymer composition of *Pseudomonas* and *Putida* growing in suspended and attached cultures. In *The Proceedings of Second International Conference on Micro- organisms in Activated Sludge and Biofilm Processes*, July 21-23, Berkeeley, CA, USA, eds D. Jenkins and S. W. Hermanowicz, pp. 555±558.

93. Jenkins D., Richard M. G., and Daigger G. T., Manual on the Causes and Control of Activated Sludge Bulking and Foaming Control. 2nd ed. Lewis Publishers Inc., USA, 193 pages, 1993.

94. Jin B., Wilen B. M. and Lant P. (2003). A comprehensive insight into floc characteristics and their impact on compressibility and settleability of activated sludge. *Chem. Eng. J.*, **95**, 221-234.

95. Jin B., Wilen B. M. and Lant P. (2004). Impacts of morphological, physical and chemical properties of sludge flocs on dewaterability of activated sludge. *Chem. Eng. J.*, **98**, 115-126.

96. Jorand F., Guicherd P., Urbain V., Manem J. and Block J. C. (1994). Hydrophobicity of activated sludge flocs and laboratory-grown bacteria. *Wat. Sci. Tech*., **30**(11), 211-218.

97. Jorand F., Zartarian F., Thomas F., Bloc J. C., Bottero J. Y., Villemin G., Urbain V. and Manem J. (1995). Chemical and structural (2D) linkage between bavteria within activated sludge flocs. *Water Res*., **29**, 1639-1647.

98. Jorand F., Boue-Bigne F., Block J. C. and Urbain V. (1998). Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances. *Wat. Sci. Tech*., **37** (4-5), 307-315.

99. Kaewpipat K. and Grady Jr CPL. (2002). Population dynamics in laboratory-scale activates sludge reactors. *Wat. Sci. Tech*., **46**(1-2), 19-27.

100. Kakii K., Kitamura S., Shirakashi T. and Kuriyama H. (1985). Effects of calcium ion on sludge characteristics. *J. Ferment Technol*., **63**(3), 263-270.

101. Kakii K., Nakatani K., Shirakishi T. and Mitsuo K. (1989). Extracellular polymers in relation to settling properties of activated sludge. *J. Ferment And Bioeng*., **68**(5), 365-370.

102. Kappeler J., and Gujer W. (2002). Verification and application of a mathematical model for 'aerobic bulking'. *Water Res*., **28**(2), 311-322.

103. Karr P. R. and Keinath T. M. (1978). Influence of particle size on sludge dewaterability. *J. Water Pollut. Control Fed.* **50**(8), 1911-1930.

104. Keiding K. and Nielsen P. H. (1997). Desorption of organic macromolecules from activated sludge:Effect of ionic composition. *Wat. Res*., **31**(7), 1665-1672.

105. Kenne, L. and Lindberg, B., Bacterial polysaccharides. In The Polysaccharides, Vol. 2, ed G. O. Aspinal. NY, USA: Academic, 1983.

106. Khoshmanesh A., Hart B.T., Duncan A., and Beckett R. (2002). Luxury uptake of phosphorus by sediment bacteria. *Water Research,* **36**, 774-778.

107. Kornberg A. (1995). Inorganic polyphosphate: toward making a forgotten polymer unforgettable. *J. Bacteriol.,* 177, 491-496.

108. Krishna C. and Loosdrecht M. C. M. (1999). Effect of temperature on storage polymers and settleability of activated sludge. *Wat. Res*., **33**(10), 2374-2382.

109. Kulaev I. S. (1979). The biochemistry of inorganic polyphosphates. Wiley, New York.

110. Lamer V. K. et al. (1957). Flocculation, subsidence and filtration of phosphate slimes. Part IV. Flocculation by gums polyelectrolytes and their influences on filtration rate. *Jour. Colloid. Sci*., **12**, 230.

111. Lamer V. K. and Healy T. W. (1963). Adsorption-flocculation rections of macromolecules at the solid-liquid interface. *Reviews of Pure and Applied Chemistry*, **13**, 112.

112. Laspidou C. S. and Rittman B. E. (2002). A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. *Wat. Res*., **36**, 2711-2720.

113. Lau A. O., Strom P. F. and Jenkins D. (1984). The competitive growth of floc forming and filamentous bacteria: a model for activated sludge bulking. *J. Water Pollut. Control Fed.,***56**, 52-61.

114. Lee C. H. and Liu J. C. (2001). Sludge dewaterability and floc structure in dual polymer conditioning. *Advances in Env. Res*., **5**, 129-136.

115. Li D. H. and Ganczarczyk J. J. (1990). Structure of activated sludge flocs. *Biotechnol. Bioeng*. **35,** 57–65.

116. Li J. (2005). Effects of Fe(III) on floc characteristics of activated sludge. *J. Chem. Technol. Biotechnol*., **80**, 313-319.

117. Liao B. Q., Allen D. G., Droppo I. G., Leppard G. G. and Liss S. N. (2001). Surface properties of sludge and their role in bioflocculation and settleability. *Wat. Res*., **35**(2), 339-350.

118. Lide D. R. (editor), Handbook of Chemistry and Physics. 71st ed., 12-1. Boston: CRC Press, 1990.

119. Liu Y. Q., Liu Y. and Tay J. H. (2004). The effects of extracellular polymeric substances on the formation and stability of biogranules. *Appl. Microbiol. Biotechnol.* **65**, 143-148.

120. Liu J. C., Lee C. H., Lai J. Y. Wang K. C., Hsu Y. C. and Chang B. V. (2001). Extracellular polymers of ozonized waste activated sludge. *Wat. Sci. Tech*., **44**(10), 137-142.

121. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951). Protein measurement with folin fenol reagent. *J. Biol. Chem.,* **193**, 265-275.

122. Machnicka A., Suschka J., and Grübel K. The importance of potassium and magnesium ions in biological phosphorus removal from wastewater. Final Report (No:12), Proceedings of Polish-Swedish Seminars, Stocholm, June 6-8, 2004 and Cracow March 17-18, 2005, Integration and optimization of urban sanitation systems.

123. Madoni P., Davoli D. and Gibin G. (2000). Survey of filamentous microorganisms from bulking and foaming activated-sludge plants in Italy. *Water Res*., **34**(6). 1767-1772.

124. Magaletti E., Urbani R., Sist P., Ferrari . R. and Cicero A. M. (2004). Abundance and chemical characterization of extracellular carbohydrates released by the marine diatom *Cylindroheca fusiformis* under N- and P-limitation. *Eur. J. Phycol*., **39**, 1-9.

125. Magara Y., Nambu S. and Utosawa K. (1976). Biochemical and physical properties of an activated sludge on settling characteristics. *Water Res*., **10**, 71-77.

126. Magnusson K. E. (1980). The hydrophobic effect and how it can be measured with relevance for cell-cell interactions. *Scand. J. Infect. Dis. Suppl*., **24**, 131-134.

127. Martinez F., Favela-Torres E. and Gomez J. (2000). Oscillations of exopolymeric composition and sludge volume indec in nitrifying flocs. *Appl. Biochem. Biotechnol*., **87**(3), 177-188.

128. Martins A. M. P., Pagilla K., Heijnen J. J. and van Loosdrecht M. C. M. (2004). Filamentous bulking sludge- a critical review. *Water Res*., **38**, 793-817.

129. McKinney R. E. and Horwood M. P. (1952). Fundamental approach to activated sludge process. I. Floc-producing bacteria. *Sew. & Ind. Wastes*, **24**, 117.

130. McKinney R. E. and Weichlein R. G. (1953). Isolation of floc-producing bacteria from activated sludge. *Appl. Microbiol.* **1**, 259.

131. McKinney R. E. (1953). Staining bacterial polysaccharides. *Jour. Bacteriol*., **66**, 453.

132. McKinney R. E. (1956). Biological flocculation. *Biol. Treat. of Sew. and Ind. Wastes*, **1**, 88.

133. McGrath J. N., Clearly S., Mullan A. and Quinn J. P. (2001). Acid-stimulated phosphate uptake by activated sludge microorganisims under aerobic laboratory conditions. *Water Res*., **35**(11), 4317-4322.

134. Metzner A. B. Non-Newtonian technology; fluid mechanics, mixing and heat transfer. In: Advances in Chemical Eng., Vol.1, Drew T. B. and Hoopes J. W. (eds.), NY: Academic Press., 1956.

135. Mikkelsen L. H. and Keiding K. (2002). Pyhsico-chemical characteristics of full scale sewage sludges with implications to dewatering. *Wat. Res*., **36**, 2451-2462.

136. Mirelman D. and Ofek I. Introduction to microbial lectins and agglutinins. In: Microbial lectins and agglutinins, D. Mirelman (ed.), NY: John Wiley & Sons, Inc., 1986.

137. Morgan J. W., Forster C. F. and Evison L. (1990). A comparative study of the nature of biopolymers extracted from anaerobic and activated sludges. *Wat. Res*., **24**(6), 743-750.

138. Murthy S. N. and Novak J. T. (1998). Effects of potassium ion on sludge settling, dewatering and effluent properties. *Wat. Sci. Tech*., **37**(4-5), 317-324.

139. Murthy S. N. (1998). Bioflocculation: Implications for activated sludge properties and wastewater treatment. Ph.D. dissertation, Dept. of Civil Eng., Virginia Polytechnic Institute and State University, USA.

140. Murthy S. N., Novak J. T. and De Haas R. D. (1998). Monitoring cations to predict and improve activated sludge settling and dewatering properties of industrial wastewaters. *Wat. Sci. Tech*., **38**(3), 119-126.

141. Murthy S. N. and Novak J. T. (1999). Factors affecting floc properties during aerobic digestion: Implications or dewatering. *Water Environ. Res.*, **71**(2), 197-202.

142. Nielsen P. H., Jahn A. and Palmgren R. (1997). Conceptual model for production and composition of exopolymers in biofilms. *Wat. Sci. Tech*., **36**(1), 11-19.

143. Nielsen P. H. and Keiding K. (1998). Disintegration of activated sludge flocs in presence of sulfide. *Water Res*., **32**, 313-320.

144. Norberg A. B. and Enfors O. (1982). Production of extracellular polysaccharide by *Zooglea ramigera*. *Appl. Environ. Microbiol*., **44**, 1231-1237.

145. Novak J. T. and Haugan B. E. (1981). Polymer extraction from activated sludge. *J. Water Pollut. Control Fed.*, **53**, 1420-1424.

146. Novak J. T., Sadler M. E. and Sudhir N. M. (2003). Mechanisms of floc destruction during anaerobic and aerobic digestion and the effect of conditioning and dewatering of biosolids. *Wat. Res*., **37**, 3136-3144.

147. Nunez C., Leon R., Guzman J., Espin G. And Soberon-Chavez G. (2000). Role of *Azotobacter vinelandii mucA and mucC* gene products in alginate production. *J. Bacteriol*., **182**, 6550–6556.

148. Örmeci B. and Vesilind P. A. (2000). Development of an improved synthetic sludge: A possible surrogate for studying activated sludge dewatering characteristics. *Wat. Res*., **34**(4), 1069-1078.

149. Özsoy G., Dilek F. B. and Sanin F. D. (2005). 'Development of a technique for extraction of heavy metals from sludge using a microwave digestion system,' presented at the 1st National Sludge Symposium, İzmir, March 23-25, 2005.

150. Painter H. A. (1973). Organic compounds in solution in sewage effluents. *Chem. Ind.* 818-822.

151. Palm J. C., Jenkins D. and Parker D. S. (1980). Relationship between organic loading, dissolved oxygen concentration and sludge settleability in the completely mixed activated sludge process. *J. Water Pollut. Control Fed.,* **52**, 2484-506.

152. Parkin G. F. and McCarty P. L. (1981). A comparison of the characteristics of soluble organic nitrogen in untreated and activated sludge treated wadtewater. *Water Res*., **15**, 139-149.

153. Patterkine V. M. (1991). The role of metals in enhanced biological phosphorus removal from wastewater. Ph. D. Thesis, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

154. Pattarkine V. M. and Randall C. W. (1999). The requirement of metal cations for enhanced biological phosphorus removal by activated sludge. *Wat. Sci. Tech*., **40**(2), 159-165.

155. Pavoni J. L., Tenney M. W. and Echelberger W. F. (1972). Bacterial exocellular polymers and biological flocculation. *J. Water Pollut. Control Fed.*, **44**, 414-431.

156. Peng Y., Gao C., Wang S., Ozaki M., and Takigawa A. (2003). Non-filamentous sludge bulking caused by a deficiency of nitrogen in industrial wastewater treatment. *Wa. Sci. and Tech.,* **47**(11), 289-295.

157. Pere J., Alen R., Viikari L. and Eriksson L. (1993). Characterization and dewatering of activated sludge from the pulp and paper industry. *Wat. Sci. Tech*., **28**(1), 193-201.

158. Pernelle J. J., Gaval G., Cotteux E. and Duchene P. (2001). Influence of transient substrate overloads on the proliferation of filamentous bacterial populations in an activated sludge pilot pilant. *Water Res*., **35**(1), 129-134.

159. Pike E. B. and Curds C. R., The microbial ecology of activated sludge systems. In: Sykes G, Skinner FA, Eds. Microbial aspects of pollution. London: Academic Press, 1971.

160. Pipes, W. O. (1979). Bulking, deflocculation, and pin-point floc. *J. Water Pollut. Control Fed.,* **51**, 62.

161. Ramothokang T. R., Drysdale G. D. and Bux F. (2003). Isolation and cultivation of filamentous bacteria implicated in activated sludge bulking. *Water SA,* **29**(4), 405-410.

162. Rickard L. F. and McClintock S. A. (1992). Potassium and magnesium requirements for enhanced biological phosphorus removal from wastewater. *Wat. Sci. Tech*., **26**, 2203-2206.

163. Ries H. E., Jr., and Meyers B. L. (1968). Flocculation mechanism, charge neutralization and bridging. *Science*, **160**, 3835, 1449.

164. Rimkus R. R. and Heil W. R. (1975). The rheology of plastic sewage sludge. The 2nd National Conference on Complete Water Reuse: Water's Interface with Energy, Air and Solids, Proceedings, Chicago, IL, May 4-8.

165. Rittman B. E. and McCarty P. L., Environmental Biotechnology: Principles and Applications. USA: McGrraw-Hill Companies, Inc., 743 pages, 2001.

166. Rosenberg M., Gutnick D. and Rosenberg E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity. *FEMS Microbial Letters*, **9**, 29-33.

167. Röske I., Bauer H. D. and Uhlmann D. (1989). Detection of phosphorus accumulating bacteria in activated sludge by electron microscopy and X-ray spectroscopy. *GWF-Wasser/Abwasser* **130**(2), 73-75.

168. Rudd T., Sterrit R. M. and Lester J. W. (1983). Extraction of extracellular polymers from activated sludge. *Biotechnol. Lett*., **5**, 39-44.

169. Salehizadeh H., Vossoughi M. and Alemzadeh I. (2000). Some investigation of bioflocculation producing bacteria. *Biochem. Eng*., **5**, 39-44.

170. Sanin F. D. and Vesilind P. A. (1994). Effects of centrifugation on the removal of extracellular polymers and physical properties of activated sludge. *Wat. Sci. Tech*., **30**(8), 117-127.

171. Sanin F. D. (1996). Bioflocculation of activated sludge: The function of extracellular polymers and calcium ions. Ph.D. dissertation, Dept. of Environmental and Civil Eng., Duke University, USA.

172. Sanin F. D. and Vesilind P. A. (1996). Synthetic sludge: a physical/chemical model in understanding bioflocculation. *Water Environ. Res.*, **68**(2), 927-933.

173. Sanin F. D. and Vesilind P. A. (2000). Bioflocculation of activated sludge: The role of calcium ion and extracellular polymers. *Env. Tech*., **21**, 1405-1412.

174. Sanin F. D. (2002). Effect of solution physical chemistry on the rheological properties of activated sludge. *Water SA*, **28**(2), 207-211.

175. Sanin S. L., Sanin F. D. and Bryers J. D. (2003). Effect of starvation on the adhesive properties of xenobiotic degrading bacteria. *Process Biochemistry*, **38**(6), 909-914.

176. Schlegel H. G. Allgemeine Mikrobiologie, Stuttgard, FRG: Georg Thieme Verlag, 1985.

1787 Schönborn C., Bauer H.D., and Röske I. (2001). Stability of enhanced biological phosphorus removal and composition of polyphosphate granules. *Water Res.,* **35**(13), 3190-3196.

178. Schuler A. J., Jenkins D. and Ronen P. (2001). Microbiaş storage products, biomass density, and settling properties of enhanced biological phosphorus removal activated sludge. *Wat. Sci. Tech*., **43**(1), 173-180.

179. Sesay M. L. and Sanin F. D. (2004). An investigation of activated sludge floc structure in relation to solids retention time. *J. Residual Sci. and Tech.* 1(2), 125.

180. Seufferheld M., Vieira M.C.F., Ruiz F. A., Rodriguez C. O., Moreno S. N. J., and Docampo R. (2003). Identification of organelles in bacteria similar to acidocalcisomes of unicellular eukaryotes. *J. Biol. Chem.,* **278**(32), 29971-29978.

181. Sezgin M., Jenkins D. and Parker D. S. (1978). A unified theory activated sludge bulking. *J. Water Pollut. Control Fed.*, **50**, 362-381.

182. Sezgin M. (1982). Variation of sludge volume index with activated sludge characteristics. *Water Res*., **16**, 83-88.

183. Sezgin M., Jenkins D. and Palm J. C. (1980). Floc size, filament length and settling properties of prototype activated sludge plants. *Prog. Water Technol*., **12**, 171-182.

184. Sheintuch M., Lev O., Einav P. and Rubin E. (1986). The role of exocellular polymer in the design of activated sludge. *Biotechnol. Bioeng*., **28**(10), 1546-1576.

185. Shin H. S., Kang S. T. and Nam S. Y. (2001). Effect of carbohydrate and protein in the EPS on sludge settling characteristics. *Wat. Sci. Tech*., **43**(6), 193-196.

186. Shimizu N. and Odawara Y. (1985). Floc-forming bacteria isolated from activated sludge in high BOD loading treatment. *J. Ferment. Technol*., **63**, 67.

187. Shiyoma M. and Toriyama A. (1985). Yields of materials extracted from activated sludge by sodium hydroxide solution and its relationship to specific resistance to filtration. *J. Jpn. Sew. Works Assoc*., **22**, 22-85.

188. Slatter P. T. (1997). The rheological characterization of sludges. *Wat. Sci. Tech*., **36**(11), 9-18.

189. Sobeck D. C. and Higgins M. J. (2002). Examination of three theories for mechanisms of cation-induced bioflocculation. *Water Res*., **36**, 527-538.

190. Somiya I., Tsuno H. and Matsumoto M. (1986). Phosphorus release-storage reaction and organic substrate behavior in biological phosphorus removal. *Water Res.,* **22**(1), 49-58.

191. Somiya I., Tsuno H. and Nishikawa M. (1987). Behaviouer of phosphorus and metals in the anaerobic-anoxic activated sludge process. In *biological phosphate removal from wastewater*. Proceedings of an IAWPRC Specialized Conference, ed. R. Ramadori. Pergamon Press, Rome, Italy.

192. Sponza D. (2002). Extracellular polymer substances and physicochemical properties of flocs in steady- and unsteady-state activated sludge systems. *Process Biochem.*, **37**, 983-998.

193. Sponza D. T. (2003). Investigation of extracellular polymer substances (EPS) and physicochemical properties of different activated sludge flocs under steady-state conditions. *Enzyme and Microbial Technology*, **32**, 375-385.

194. Sponza D. T. (2004). Properties of four biological flocs as related to settling. *J. Env. Eng.* **130**(11), 1289-1300.

195. Steiner A. E., McLaren D. A. and Forster C. F. (1976). The nature of activated sludge flocs. *Water Res*., **10**, 25-30.

196. Sudhir N. M. (1998). Bioflocculation: Implications for activated sludge properties and wastewater treatment. Ph.D. dissertation, Virginia Polytechnic Institute and State Univ., Blacksburg, Va.

197. Sutherland I. W. Bacterial exopolysaccharides- their nature and production In: *Surface carbohydrates of the prokaryotic cell*, I. W. Sutherland (ed.) London: Academic Press, 1977.

198. Sutherland I. W. Biotechnology of microbial exopolysaccharides. Cambridge: Cambridge University Press, 1990.

199. Takeda, M., Ishigami, M., Shimada, A., Matsuoka H. and Nakamura I. (1994). Separation and preliminary characterization of acidic polysaccharides produced by *Enterobacter* sp. *J. Fermen. Bioeng.*, **78,** 140.

200. Tchobanoglous G. and Burton F. L., Wastewater Engineering: Treatment, Disposal and Reuse. NY, USA: McGraw Hill, 1334 pages, 1991.

201. Tenney M. W. and Stumn W. (1965). Chemical flocculation of microorganisms in biological waste treatment. *J. Water Pollut. Control Fed.*, **37**, 1370-1388.

202. Tenney M. W. and Verhoff, F. H. (1973). Chemical and autoflocculation of microorganisms in biological wastewater treatment. *Biotechnol. Bioengrg*., **15**, 1045-1073.

203. Tezuka Y. (1967). Magnesium ion as a factor governing bacterial flocculation. *Appl. Microbiol.*, 15, 1256.

204. Tezuka Y. (1969). Cation-dependent flocculation in a *Flavobacterium* species predominant in activated sludge. *Appl. Microbiol.*, **17**, 222-226.

205. Thompson G. and Forster C. Bulking in activated sludge plants treating paper mill wastewaters. (2003). *Water Res.,* **37**, 2636-2644.

206. Turtin İ., Vatansever A. and Sanin F. D. (2005). 'Phosphorus deficiency and sludge bulking problem,' presented at the 1st National Sludge Symposium, İzmir, March 23-25, 2005.

207. Urbain V., Block J. C. and Manem J. (1993). Bioflocculation in activated sludge: An analytic approach. *Wat. Res*., 27(5), 829-838.

208. Valin S. D. and Sutherland D. J. (1982). Predicting bioflocculation: new developments in the application of flocculation theory. *Environ. Tech. Lett*., **3**, 363-374.

209. Vesilind P. A., Treatment and Disposal of Wastewater Sludge. Ann Arbor, USA: Ann Arbor Science Publishers Inc., 1979.

210. Vesilind P.A. (1994). The Role of Water in Sludge Dewatering. *Water Environ. Res*., **66** (1), 4-11.

211. Wanner J. (1992). Comparison of biocenoses from continuous and sequencing batch reactors. *Wat. Sci. Tech*., **25**(6), 239-249.

212. Wanner J. (1994). *Activated Sludge Bulking and Foaming Control.* Technomic Publishing Company, Inc., USA, 324 pages.

213. Wilen B. M., Jin B. and Lant P. (2003a). The influence of chemical constituents in activated sludge on surface and flocculating properties. *Wat. Res*., **37**, 2127-2139.

214. Wilen B. M., Jin B. and Lant P. (2003b). Relationship between flocculation of activated sludge composition of extracellular polymeric substances. *Wat. Sci. Tech*., **47**(12), 95-103.

215. Wilkinson J. F. (1958). The extracellular polysaccharides of bacteria. *Bacterial Rev*., **22**, 46-73.

216. Wu Y. C., Smith E. D. and Novak R. (1982). Filterability of activated sludge in response to growth conditions. *J. Water Pollut. Control Fed.* **54**(5), 444-456.

217. Yang P. Y. and Chen Y. L. (1977). Operational characteristics and biological kinetic constants of extended aeration process. *J. Water Pollut. Control Fed.*, **49**, 678-688.

218. Yurteri R. G. (1982). Bacterial Desulfurization of Turkish Lignites. M. Sc. Dissertation, Dept. of Environmental Eng. Middle East Technical University, Turkey.

219. Zita A. and Hermansson M. (1994). Effects of ionic strength on bacterial adhesion and stability of flocs in a wastewater activated sludge system. *Appl. Environ. Microbiol*., **60**, 3041-3048.

220. Zita A. and Hermansson M. (1997). Effect of bacterial cell surface Structures and hydrophobicity on attachment to activated sludge flocs. *Appl. Eviron. Microbiol*., **63**, 1168-1170.

APPENDIX A

CALIBRATION CURVES

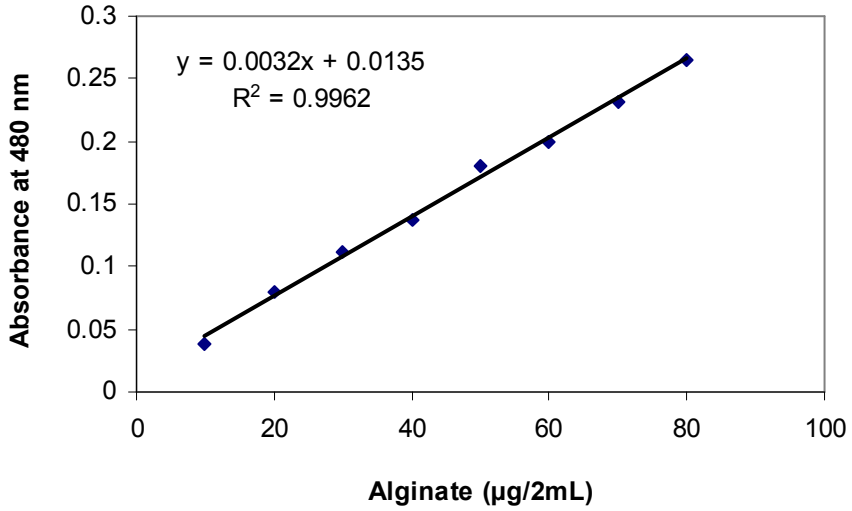


Figure A.1. Calibration curve for carbohydrate measured by Dubois method

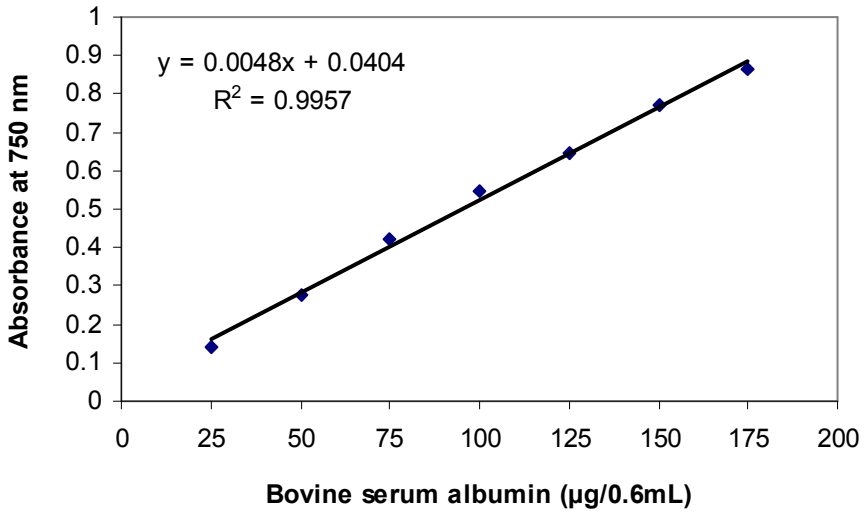


Figure A.2. Calibration curve for protein measured by Lowry method

APPENDIX B

GRAPHS USED FOR THE DETERMINATION OF SPECIFIC RESISTANCE TO FILTRATION (SRF)

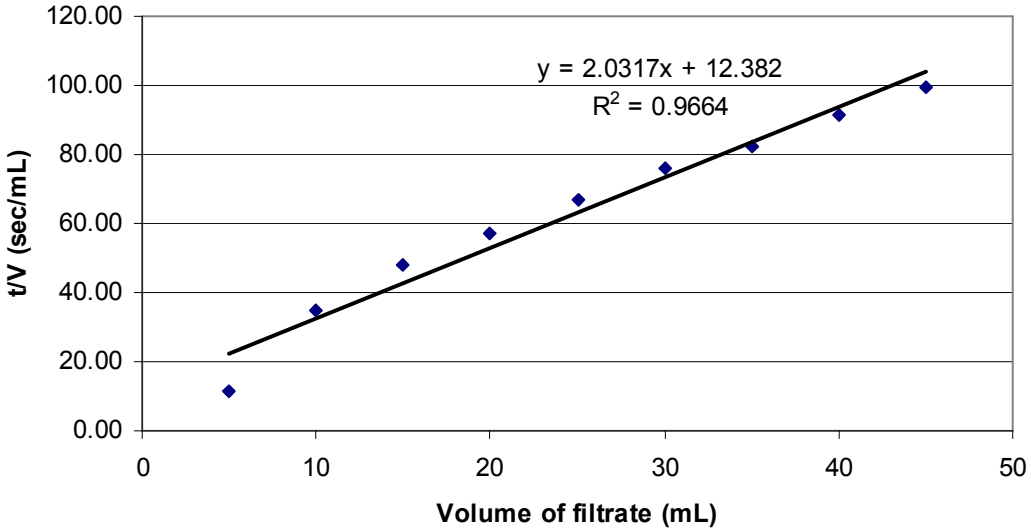


Figure B.1. t/V vs. V graph for control reactor (Ca = 0.27 meq/L)

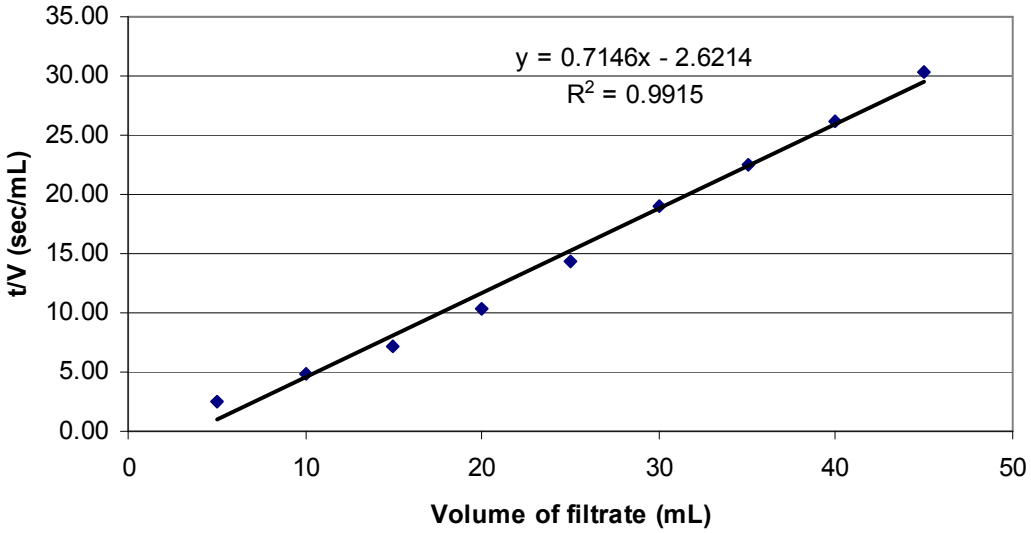


Figure B.2. t/V vs. V graph for reactor 5 (Ca = 5 meq/L)

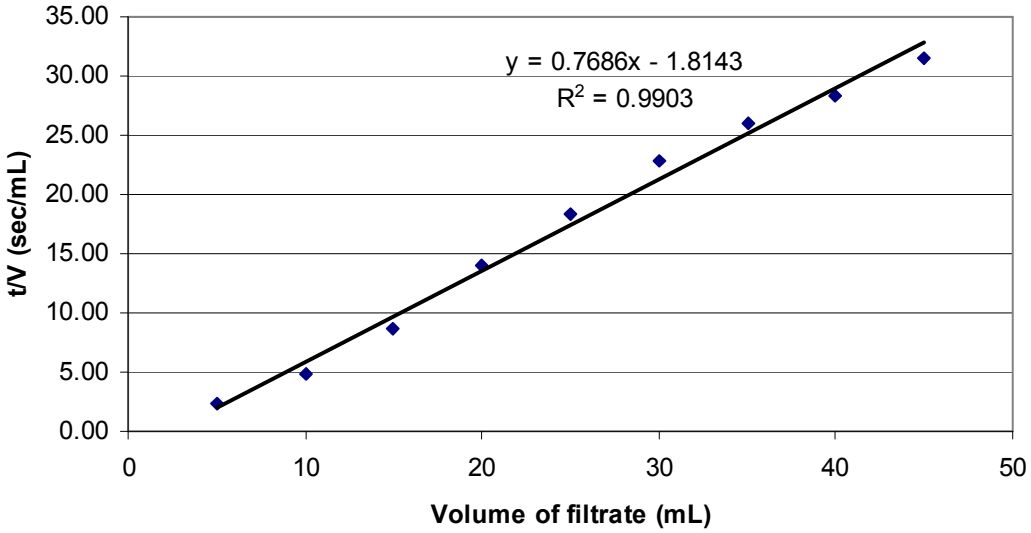


Figure B.3. t/V vs. V graph for reactor 6 (Ca = 10 meq/L)

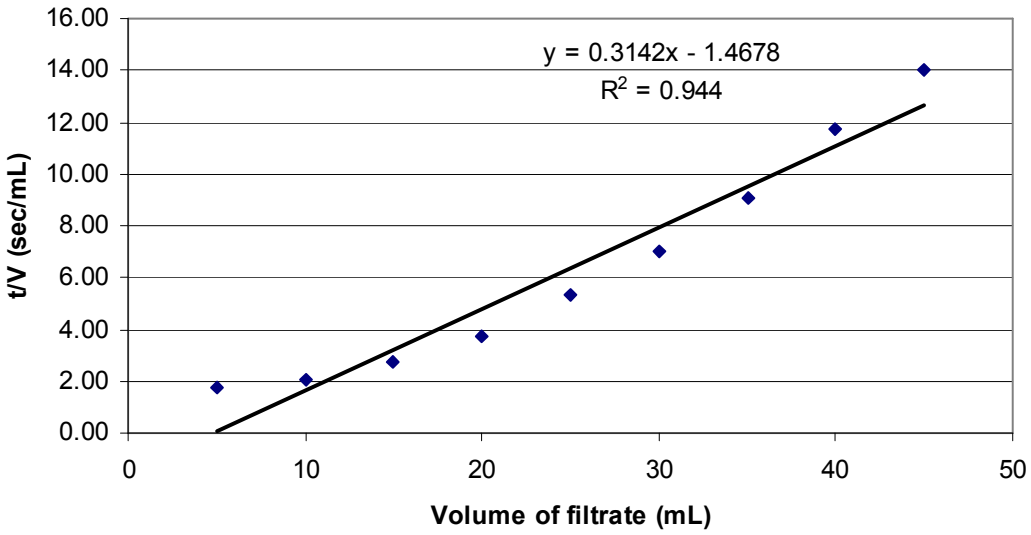


Figure B.4. t/V vs. V graph for reactor 7 (Ca = 20 meq/L)

APPENDIX C

ZONE SETTLING VELOCITIES OF THE REACTORS

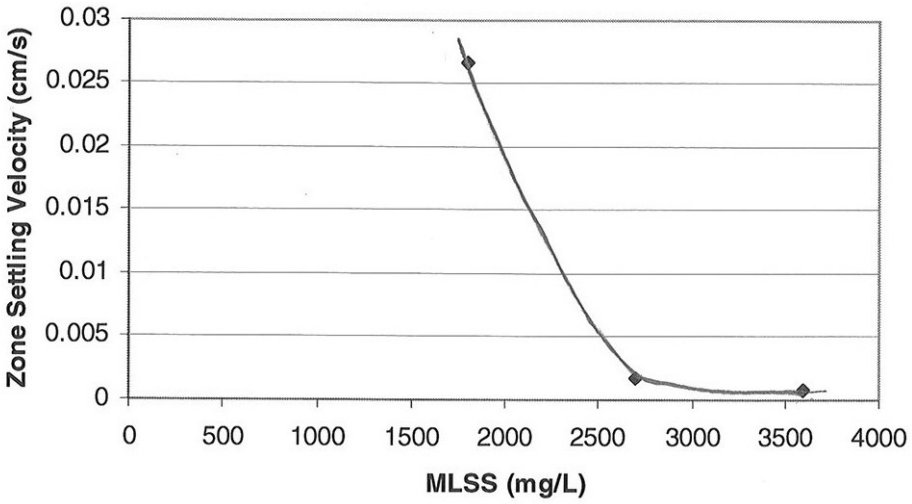


Figure C.1. Zone settling velocity of control reactor (Ca = 0.27 meq/L) with respect to MLSS concentration

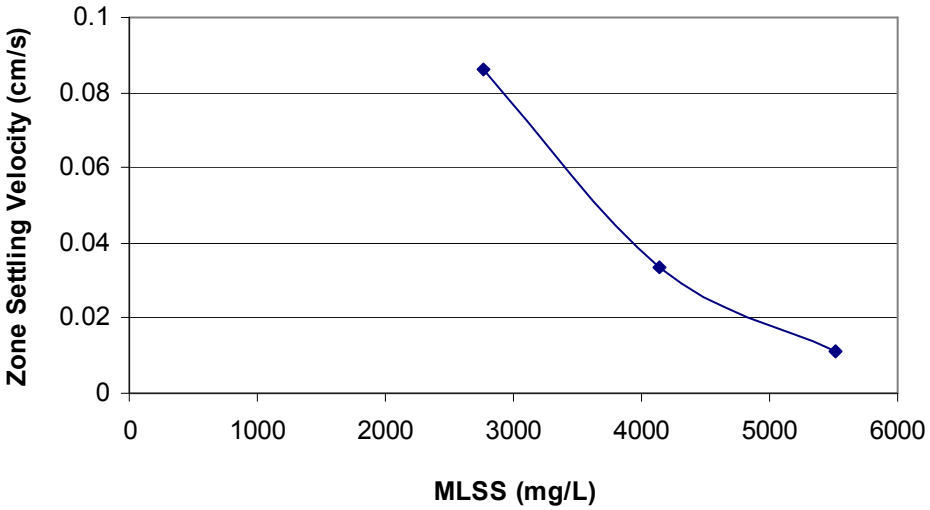


Figure C.2. Zone settling velocity of reactor 5 (Ca = 5 meq/L) with respect to MLSS concentration

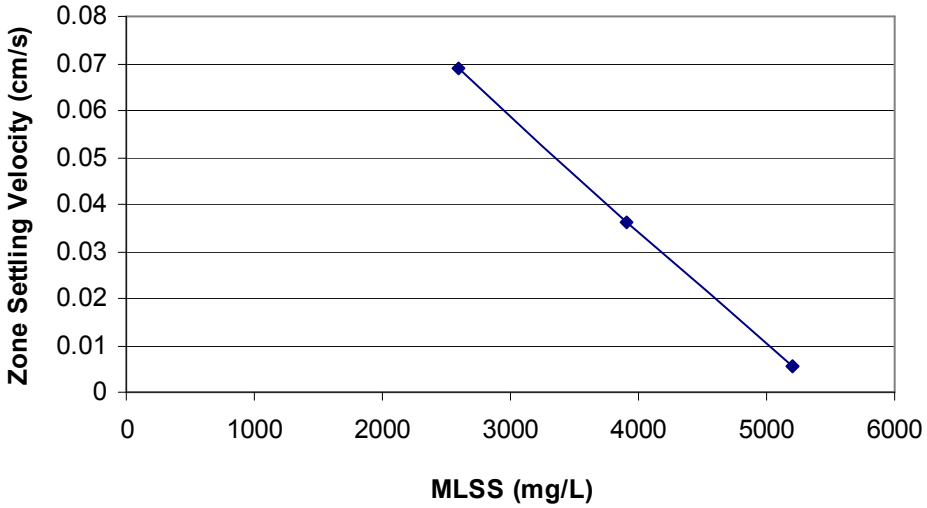


Figure C.3. Zone settling velocity of reactor 6 (Ca = 10 meq/L) with respect to MLSS concentration

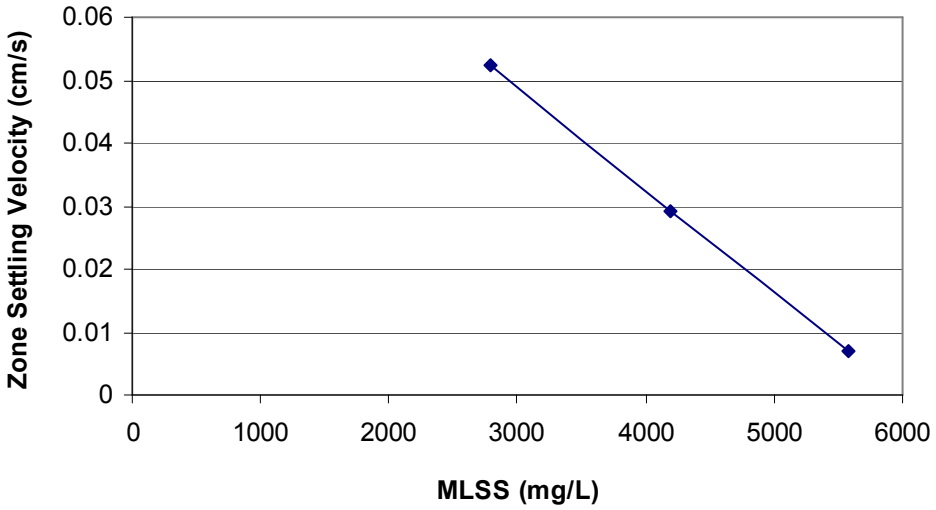


Figure C.4. Zone settling velocity of reactor 7 (Ca = 20 meq/L) with respect to MLSS concentration