RELATIONSHIP BETWEEN METAL INHIBITION AND MICROBIAL PRODUCTS IN BIOLOGICAL SYSTEMS

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dedicated to my son…

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RELATIONSHIP BETWEEN METAL INHIBITION AND MICROBIAL PRODUCTS IN BIOLOGICAL SYSTEMS

One of the main topics in biological treatment systems is the production of extracellular polymeric substances (EPS) on surface of cells. These substances consisting mainly of proteins and carbohydrates have the ability to bind metals. In this dissertation, first activated sludge reactors were operated in long term at different COD/TKN ratios and with different organic substrates. In each sludge EPS production as well as the changes taking place in EPS were examined. The study pointed to the existence of a very loosely bound EPS fraction in sludge of which there was no mention in literature before. In addition, the study examined the relationship between EPS and surface charge and hydrophobicity of sludges. The results indicated that in an activated sludge system wastewater composition was of crucial importance and the composition of EPS varied with the feed supplied to an activated sludge system. The inhibitory effect and binding of silver and nanosilver (AgNP) was studied. AgNP was synthesized in laboratory. Although the concentration of nanosilver was raised to levels above the normal concentrations found in sewage, nanosilver had no effect on substrate removal since it lost its stability. However, the presence of nanosilver clearly affected the structure of EPS. The binding of nanosilver to the various fractions in EPS is of importance since this determines the transfer of nanosilver to the receiving water or accumulation in sludge phase. Overall, the study revealed that “heavy metal inhibition” depended on many factors.

BİYOLOJİK SİSTEMLERDE MİKROBİYEL ÜRÜNLER VE METAL İNHİBİSYONU

Biyolojik arıtma sistemlerinde üzerinde önemle durulan konulardan birisi, hücre yüzeyinde EPS (extracellular polymeric substances) olarak bilinen hücre dışı polimerik maddelerin oluşmasıdır. Daha çok protein ve karbonhidratlardan oluşan bu maddeler çeşitli atıksulardaki metalleri bağlama kapasitesine sahiptirler. Bu çalışmada öncelikle değişik KOI/TKA oranlarında ve çeşitli organik sübstratlarla beslenen aktif çamurlarda uzun süre zarfında organik karbon giderimi ve nitrifikasyon incelenmiş, buna paralel olarak EPS oluşumu ve değişimi detaylı olarak araştırılmıştır. Aktif çamurdan literatürde daha önce bahsedilmeyen çok zayıf bağlı bir EPS fraksiyonu ekstre edilmiştir. Ayrıca EPS'in kompozisyonu ve fraksiyonları ile çamurun yüzey yükü ve hidrofobisitesi arasındaki bağlantı da araştırılmıştır. Çalışmada aynı zamanda biyolojik arıtma sistemleri açısından önemli bir metal olan gümüş ve nanogümüşün (AgNP) inhibitör etkisi ve gümüşün EPS fraksiyonlarına bağlanması incelenmiştir. Ayrıca, laboratuvar ortamında sentezlenen AgNP'nin de aktif çamur üzerindeki inhibitör etkisi ve EPS fraksiyonlarına etkisi araştırılmıştır. Sonuçlar, biyolojik arıtma sırasında atıksu kompozisyonunun çok önemli bir parametre olduğunu, değişik şekillerde beslenen çamurların EPS içeriğinin değiştiğini göstermektedir. Aktif çamura sürekli nanogümüş eklenmesi halinde, nanogümüş stabilitesini kaybettiği için normalde evsel atıksuda görülen konsantrasyonların da üzerine çıkıldığında sübstrat giderimi inhibe olmamaktadır. Öte yandan bu nanopartikül EPS yapısını değiştirmektedir. Nanogümüşün EPS'in çeşitli fraksiyonlarına bağlanması alıcı ortama taşınması veya çamurda birikimi açısından önem taşımaktadır. Çalışmadan çıkan sonuçlara göre, metal inhibisyonunun birçok faktöre bağlı olan göreceli bir kavram olduğu gösterilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Units used
μ	Specific Growth Rate	(1/d)
θ	Hydraulic Retention Time	(d)
θ_x	Sludge Age	(d)
AAS	Atomic Absorption Spectroscopy	
Ag^+	Free Silver Ion	(mg/L)
Ag^0	Metallic Silver	
AgNP	Nanosilver	
Ag_2S	Silver Sulfide	
AOB	Ammonia Oxidizing Bacteria	
ASV	Anodic Stripping Voltammetry	
ATU	N-Allylthiourea	(mg/L)
b	Decay Coefficient of Microorganisms	(1/d)
BAP	Biomass-Associated Products	
BNR	Biological Nutrient Removal	
BOD	Biochemical Oxygen Demand	(mg/L)
BPEI	Branched Polyethylenimine	
BSA	Bovine Serum Albumin	
C/N	Carbon to Nitrogen ratio	
C-CO_2	Carbonaceous CO_2 production	(mg)
COD	Chemical Oxygen Demand	(mg/L)
CPE	Carbon Paste Electrode	
CR	Control Reactor	
CR_{AgNP}	Control Reactor receiving AgNP	
CSTR	Continuously Stirred Tank Reactor	
DME	Dropping Mercury Drop Electrode	
DNP	2,4-Dinitrophenol	
DO	Dissolved Oxygen	(mg/L)
DOM	Dissolved Organic Matter	

DPP	Differential Pulse Polagragphy	
DPASV	Differential Pulse Anodic Stripping Voltammetry	
EAWAG	Swiss Federal Institute of Aquatic Science and Technology	
EPS	Extracellular Polymeric Substances	
SEM	Scanning Electron Microscopy	
F/M	Food to Microorganism ratio	
GCE	Glassy Carbon Electrode	
GPC	Gel-Permeating Chromatography	
HD	Sludge Hydrophobicity	
HDME	Hanging Mercury Drop Electrode	
HPSEC	High Pressure Size Exclusion Chromatography	
HRT	Hydraulic Retention Time	
IC_{50}	Inhibitor Concentration Causing 50% Inhibition	
ICP	Inductively Coupled Plasma	
IUPAC	International Union of Pure and Applied Chemistry	
K_{sp}	Solubility Products	
LB-EPS	Loosely Bound-Extracellular Polymeric Substances	
MBR	Membrane Bioreactor	
MCRT	Mean Cell Residence Time	
MLSS	Mixed Liquor Suspended Solids	(mg/L)
MLVSS	Mixed Liquor Volatile Suspended Solids	(mg/L)
MW	Molecular Weight	
NH_4-N	Ammonium Nitrogen	(mg/L)
NOM	Natural Organic Matter	
OD	Optical Density	
OUR	Oxygen Uptake Rate	(mg/L/h)
PBS	Phosphate Buffer	
P/C	Protein to Carbohydrate Ratio	
PP	Pulse Polarography	
PVP	PolyVinylpyrrolidone	
R1	Reactor 1	
R2	Reactor 2	
R3	Reactor 3	

RDE	Rotating Disc Electrode	
RG	Glucose Reactor	
RP	Peptone Reactor	
RP_{AgNP}	Peptone Reactor receiving AgNP with Feed	
SBR	Sequencing Batch Reactor	
SC	Sludge Surface Charge	
SCFB	Semi-Continuously Fed Batch (Reactor)	
SEC	Size Exclusion Chromatography	
SEM	Scanning Electron Microscopy	
S-EPS	Soluble-Extracellular Polymeric Substances	
SMP	Soluble Microbial Products	
Sp-EPS	Supernatant- Extracellular Polymeric Substances	
SRT	Solids Retention Time	(d)
SS	Suspended Solids	(mg/L)
SSE	Solid State Electrode	
TB-EPS	Tightly Bound-Extracellular Polymeric Substances	
TEM	Transmission Electron Microscopy	
TKN	Total Kjeldahl Nitrogen	(mg/L)
TMFE	Thin Mercury Film Electrode	
UAP	Utilization-Associated Products	
WWTP	Wastewater Treatment Plant	
VA	Voltammetry	
VLB-EPS	Very Loosely Bound-EPS	
VSS	Volatile Suspended Solids	(mg/L)
X_a	Active Biomass Concentration	(mg/L)
Y_h	Yield Coefficient of Heterotrophic Bacteria	(mg/mg)

1. INTRODUCTION

The activated sludge process treats sewage and industrial wastewaters using air and a biological floc composed of bacteria and protozoa. In an activated sludge system, microorganisms are embedded in a matrix called extracellular polymeric substances (EPS). Microbial EPS are biosynthetic polymers (biopolymers) which keep microbial aggregates together in a three-dimensional gel-like hydrated matrix through weak physicochemical interactions such as electrostatic, hydrophobic, van der Waals and hydrogen-bonding (Zartarian et al., 1997; González-Brambila et al., 2006; Leone et al., 2006).

EPS have important properties from the point of engineering. For example, the EPS have ability to remove toxic organic compounds from different types of wastewaters, sludge and soils. The concentration and characteristics of EPS are the important parameters which affect sludge dewatering, flocculation and settling (Houghton et al., 2001; More et al., 2014). Moreover, Zouboulis and Katsoyiannis (2004) found that the application of EPS from *Rhizomonas sp.* was quite efficient (when compared with alum) in the removal of humic acids from synthetic solutions as well as in the reduction of chemical oxygen demand (COD) (45%) from landfill leachate.

There may be many factors affecting EPS production such as solids retention time (SRT), pH, the food/microorganism ratio (F/M), the ratio of carbon to nitrogen (C/N), the type of organic carbon etc. One of the methods to control EPS is to monitor the nutrients in feed wastewater (Hoa et al., 2003). The nature and concentration of nutrients affect biodegradation of organic waste. Nutrients are necessary components for the growth of bacteria and they can also stimulate the production of the surface biopolymers called EPS.

Combination of industrial and municipal wastewaters for treatment in sewage plants increases the possibility of contamination of the influent with metal ions which might reach inhibitory levels. In that case, wastewater treatment processes can be influenced since microorganisms are negatively affected in the presence of metals. Microorganisms produce EPS to protect themselves from the inhibitory effect of metals and xenobiotics.

Since microorganisms are the key components for the decomposition of organic matter, the effect of metal toxicity on microorganisms has received special attention in recent years.

In this study, in order to investigate EPS production under different nutritional conditions, eight different activated sludge reactors were operated. Until now, there are few studies focusing on the effect of nutrient balance (COD:TKN) on the production and composition of EPS and the response of different sludges to toxic metals. The toxic effects of silver (Ag^+) and nanosilver (AgNP) were studied under different nutritional conditions. Heavy metal inhibition tests were carried out both in short- and long-term. In parallel to this, EPS production was examined. Complexation of EPS with heavy metals was studied using voltammetric measurements.

2. AIM AND SCOPE OF THE STUDY

This study was designed to investigate the production of EPS in different activated sludge systems fed with different types of synthetic wastewaters. For this purpose, semi-continuously fed batch (SCFB) activated sludge reactors were used.

Initially, three activated sludge reactors were operated at COD/TKN ratios, of 10, 5 and 0 while each represented a different treatment system. The COD/TKN ratio of 10 represented the conditions in an conventional activated sludge treating municipal wastewater, whereas the reactor operated at a COD/TKN ratio of 5 represented a carbon-limited case. Also, another reactor was operated at the COD/TKN ratio of zero to represent a separate-stage nitrification system. The aim was to show the effect of the COD/TKN ratio on EPS production. There is a lack of information on how EPS change in a system combining organic carbon removal and nitrification. In particular, there is a lack of understanding on how sludges produce EPS when they are enriched in terms of nitrifiers.

In the next step, three other reactors were operated at a constant COD/TKN ratio of 10. However, in this case each reactor received a different organic carbon source. The aim was to observe the effect of substrate type on EPS production. One of the reactors received a typical sewage. The others were fed with proteinaceous and carbonaceous wastewaters. Changes in EPS composition and surface characteristics of sludges were studied.

Following this, EPS production was also monitored when these activated sludges were exposed to heavy metals. In wastewater treatment systems, metals are able to complex with organic and inorganic compounds in liquid phase. Moreover, EPS have the ability to bind to metals. The main aim was to determine the toxicity of heavy metals, in the form of Ag^{+} and Nanosilver particles (AgNP), on these different biological sludges. Results were interpreted together with EPS findings.

Thus the scope of this study can be summarized as follows:

- Examination of EPS production in different activated sludge systems.
- Examination of the influence of inhibitory heavy metals on EPS production.
- Studying the affinity of different EPS fraction to heavy metals.
- Examination of the toxic behavior of Ag^{+} and AgNP on activated sludge in short-term.
- Examination of EPS production in the case of long-term exposure to nanosilver (AgNP).

3. THEORETICAL BACKGROUND

3.1. Activated sludge

Activated sludge consists of biological flocs that are composed of microorganisms, nonliving organic matter and inorganic materials (Wang et al., 2009). The microorganisms include bacteria, fungi, protozoa, and higher forms of animals such as rotifers, insect larvae, and worms. An activated sludge process can be defined as a system in which biological flocs are continuously circulated to come into contact and to oxidize the organic substances in the presence of oxygen. The objectives of activated sludge treatment are twofold:

a) to obtain the maximum possible removal of organic substances with the shortest possible time,
b) to produce flocculant biological flocs having a good settling characteristic. Both are essential in controlling the secondary effluent quality (Wang et al., 2009).

The process of floc formation is far from being understood. Originally, it was thought that slime-forming bacteria and many other bacteria and protozoa were associated with floc formation. The exact nature of this flocculating material is still not known although it appears largely bacterial in origin. This flocculating material can be readily extracted from activated sludge (Brown and Lester, 1980) and constitutes a significant portion of the dry weight of the sludge, up to 10%. All the studies have shown that the material is a polymer, which is composed of a number of organic compounds, such as polysaccharides, amino polysaccharides, and protein (Sato and Ose, 1984). Lipids may also be present, there might also be minor amounts of nucleic acids and other biopolymers, but the exact nature of these flocculating polymers will depend on the species of bacteria or protozoa producing it (Dignac et al., 1998).

Each polymer will have varying surface properties and charges that will influence not only the settling characteristics, but also the water binding properties of the floc. The polymer does not only give the floc components cohesion, it also allows suspended

particles in the waste to bind to the floc by adsorption. Cations and anions, including phosphorus and a range of pollutants and toxic compounds, are known to be adsorbed by such polymers (Beech and Cheung, 1995; Sophie Comte et al., 2006a). Therefore, the polymer has a critical role in the operation of the activated sludge, biofilm and membrane systems (Flemming and Wingender, 2001a, 2001b).

These polymers, also referred to as EPS, are not food reserves, like poly-β-hydroxybutrate, and they are not easily decomposed. The EPS matrix is a dynamic system that enables cells in flocs to function in a manner similar to multi-cellular organisms. EPS have unique sorption properties, especially for metals. Decho (2000) has suggested that sorption of heavy metals is a strategy to protect the bacteria against toxic effects.

3.2. Factors Affecting the Activated Sludge Process

When designing treatment systems, parameters such as dissolved oxygen concentration, aeration characteristics and solid retention times and many others are significant. The effect of each parameter should be taken into consideration before designing the system. In this section the most significant parameters in activated sludge operation are briefly summarized.

3.2.1. Operational Parameters

3.2.1.1. Food to Microorganism Ratio (F/M). F/M ratio can be defined as the mass of substrate applied per unit time per mass of microorganisms contained in the aeration tank (Grady et al., 1999). Equation 3.1 shows the F/M ratio which is generally expressed in g BOD or COD/g VSS.d (Tchobanoglous et al., 2003).

$$\text{F/M Ratio} = \left(\frac{\text{total applied substrate rate}}{\text{total microbial biomass}}\right) = \frac{QS_O}{VX} \qquad (3.1)$$

where;
Q is influent wastewater flowrate, m^3/d
S_0 is influent BOD or COD concentration, g/m^3
V is the tank volume, m^3
X is mixed liquor biomass concentration in the aeration tank, g/m^3.

The F/M ratio is one of the significant design and operational parameters of activated sludge systems. A balance between substrate consumption and biomass generation helps in achieving system equilibrium. The F/M ratio is responsible for the decomposition of organic matter. The type of activated sludge system can be defined by its F/M ratio.

- Extended aeration, $0.05 < F/M < 0.15$ kg BOD_5/kg MLVSS.d
- Conventional activated sludge system, $0.2 < F/M < 0.5$ kg BOD_5/kg MLVSS.d
- Completely mixed, $0.2 < F/M < 1.0$ kg BOD_5/kg MLVSS.d
- High rate, $0.4 < F/M < 1.5$ kg BOD_5/kg MLVSS.d

3.2.1.2. The Solids Retention Time (SRT). The sludge age, also defined as solids retention time (SRT), is the most fundamental and important parameter in the design, operation and control of biological nutrient removal activated sludge systems. It is the main driver that governs the activated sludge system effluent quality, size and waste activated sludge quality (residual biodegradable organics and P content). Generally, the better the effluent and waste activated sludge quality required from the system, the longer the sludge age, the larger the biological reactor and the greater number of wastewater characteristics that need to be known (Ekama, 2010).

The SRT affects the character and condition of activated sludge flocs within the aeration basin. It is calculated as the total amount of sludge solids in the system divided by the rate of loss of sludge from the system. In practical terms, it is impossible to take into account all the sludge in the various stages of the activated sludge process, including the pipework and sedimentation tank as well as the aeration basin. Therefore, a simplified equation is used as shown in Equation 3.2.

$$\theta_x = \left(\frac{V.X}{Qe.Xe}\right) \quad (3.2)$$

where:

V is volume of liquid in the aeration tank (m^3)

X is volatile suspended solids (MLVSS) concentration (mg/L)

X_e is effluent MLVSS concentration (mg/L)

Q_e is effluent discharge rate (m^3/d)

Θ_x is SRT in days.

SRT is an operational factor giving control over sludge activity because SRT is the reciprocal of the net specific growth rate of sludge and thus can be considered as a measure of sludge activity. A low SRT (< 0.5 d) indicates a sludge with a high growth rate as used in high-rate units for pretreatment or partial treatment; a high SRT (> 5 d) indicates a low growth rate sludge, such as extended aeration systems. A disadvantage of a very short sludge age is that the predators of free bacteria (those not aggregated to flocs) do not have sufficient residence time to develop, so that the effluent quality is not very high: part of the active sludge will be discharged as free bacteria in the effluent. For that reason both biological oxygen demand (BOD) and MLVSS concentrations in the effluent will be relatively high. At longer sludge ages (above 5 to 8 days), the predators of free bacteria will develop and BOD and MLVSS concentrations can be very small (< 5 to 10 mg/L), if the final settler works properly. Conventional activated sludge has a SRT of between 3 and 4 d, and has good settling properties. However, at SRT above 6 d or between 0.5 and 3 d, there is a reduction in settleability. SRT is controlled by altering the sludge wastage rate (Gray, 2004).

If sludge wasting rate is higher than the growth rate of microorganisms, microorganisms in the reactor will be washed out from the reactor and the system fails. The minimum SRT can be calculated for activated sludge systems. The design SRT should always be greater than this minimum.

3.2.1.3. Dissolved Oxygen (DO). Generally, oxygen concentrations are below saturation due to the presence and oxidation of decaying organic matter (suspended, benthic, or sediment). In addition to the organics, nitrogenous materials may exert an oxygen demand through bacterial oxidation of ammonia to nitrate (Krenkel and Novotney, 1980). Concentrations below 5 mg/L may adversely affect function and survival of biological communities.

3.2.2. Nutritional Parameters

Nutritional parameters such as the ratio of organic carbon to nitrogen (C/N) in the influent or the type of organic substances in the activated sludge system can affect microbial metabolism. Therefore, they can also affect flocculation, settling and dewatering properties of sludge (Ye et al., 2011b).

3.2.2.1. The Ratio of Organic Carbon to Nitrogen (C/N). The C/N ratio is one of the critical factors associated with the performance of nitrification systems. Okabe et al. (1996) investigated the effects of different C/N ratios on time-dependent population dynamics of nitrifiers and heterotrophs in mixed-population biofilms as well as on nitrification efficiency. The results showed that the population dynamics and nitrification efficiency were strongly related to the initial microbial composition in the biofilms and the C/N ratio. It seems that a higher C/N ratio would retard the accumulation of nitrifying bacteria. This in turn results in a considerably long start-up period for complete and stable nitrification owing to competition for dissolved oxygen and space in the biofilms. For biological treatment process incorporating a nitrification unit, the overall solids retention time should be designed to be greater than the minimum solids retention time required for nitrification (Wang et al., 2009).

The C/N ratio can also affect the microbial physiology of sludge, thereby affecting the nature and content of EPS. In municipal wastewaters and others, the C/N ratio may change in the wastewater itself or at different stages of treatment. The effect of this ratio on EPS has been shown in a limited number of studies (Durmaz and Sanin, 2003; Yuncu et al., 2006; Ye et al., 2011a). There is still limited information on the effect of the C/N ratio on production and composition of EPS. Moreover, studies on EPS often address

simultaneous organic carbon and nitrogen removal whereas little attention is paid to separate-stage nitrification systems receiving no organic load.

In addition, the changes occurring in the production and composition of EPS are usually examined by the exposure of the same reactor to short-term changes in C/N ratio. However, in such a case EPS production might be affected by previous conditions.

The C/N ratio has also an impact on heavy metal biosorption. According to literature study, the biosorptive capacity of activated sludge was highly dependent on type of metal and the C/N ratio (Yuncu et al., 2006). Yuncu and coworkers (2006) focused on sorption of Cd(II), Zn(II), Cu(II) and Ni(II). They found that an increase in C/N ratio resulted in an increase in the Cd(II) sorption capacity of activated sludge whereas it decreased the Cu(II) sorption capacity. As for Zn(II), a different behavior was observed such that, the highest sorption capacity was observed at the C/N ratio of 21 and the lowest capacity occurred at the C/N ratio of 43. For Ni(II) biosorption, isotherm tests showed that there was not a relationship between maximum adsorptive capacity and the C/N ratio.

3.2.2.2. Substrate Type. The production and composition of EPS depend on wastewater composition and operational conditions (Knocke et al., 1993; Wilén et al., 2003a, 2008). In biological treatment systems like activated sludge, the type of organic substrate has a substantial effect on microbial community and metabolism, thereby it might indirectly influence also the production of EPS. For example, Li and Yang (2007) showed that when a sludge received glucose alone, more EPS was produced than in the case of acetate. Additionally, Sheng et al. (2006) found that a photosynthetic bacterial strain produced more EPS when fed with benzoate than acetate, propionate and butyrate. Until now, few studies compared the effect of different carbon sources, particularly in the case of mixed cultures such as activated sludge.

3.3. Overview of Extracellular Polymeric Substances (EPS)

EPS were defined by Geesey (1982) as "extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates". The abbreviation “EPS” has been used for “extracellular polysaccharides, exopolysaccharides,

exopolymers and extracellular polymeric substances". Polysaccharides have often been assumed to be the most abundant components of EPS in early biofilm research (Costerton et al., 1981). That may be the reason why the term "EPS" has frequently been used as an abbreviation for "extracellular polysaccharides" or "exopolysaccharides". However, proteins and nucleic acids (Frølund et al., 1996; Nielsen et al., 1997; Dignac et al., 1998) as well as amphiphilic compounds including (phospho)lipids (Neu, 1996) have also been shown to appear in significant amounts or even predominate in EPS preparations from activated sludges, sewer biofilms, trickling filter biofilms, and pure cultures of bacteria. In addition, some researchers described humic substances as components of EPS matrices of soil and water biofilms (Jahn and Nielsen, 1998). In later times, the abbreviation "EPS" was used as a more general and comprehensive term for different classes of organic macromolecules such as polysaccharides, proteins, nucleic acids, (phospho)lipids, and other polymeric compounds, which have been found to occur in the intercellular spaces of microbial aggregates. The production of EPS is a general property of microorganisms in natural environments and has been shown to occur both in prokaryotic (Bacteria, Archaea) and in eukaryotic (algae, fungi) microorganisms (Wingender et al., 1999).

The vast majority of microorganisms live and grow in aggregated forms such as biofilms and flocs ("planktonic biofilms"). This mode of existence is lumped in the somewhat inexact but generally accepted expression "biofilm". The common feature of all these phenomena is that the microorganisms are embedded in a matrix of extracellular polymeric substances (Wingender et al., 1999).

EPS bind with cells through complex interactions to form a vast net-like structure with plenty of water that protects cells against dewatering (Wingender et al., 1999) and the harm of toxic substances (Sutherland, 2001). Part of EPS can serve as carbon or energy sources in the case of nutrient shortage (Sutherland, 2001). EPS also accelerate the formation of microbial aggregates through binding cells (Liu et al., 2004).

3.4. Composition and Production of EPS

The variation in the composition of extracted EPS is attributed to many factors, such as culture, growth phase, reactor operation, bioreactor type, extraction method, and the

analytical tool used (Nielsen and Jahn, 1999). The content and composition of the EPS extracted from various microbial aggregates are reported to be heterogeneous (Wingender et al., 1999).

3.4.1. Dependence of EPS on Culture Type

EPS studies have been carried out using pure and mixed cultures, both in aerobic and anaerobic conditions. Depending on the type of bacterial species, in pure cultures, the efficiency of the extraction method and EPS yield can be variable. Symbiotic relationships between mixed culture microorganisms may increase substrate utilization and EPS yield. EPS studies on mixed cultures are important because such microbial relationships (e.g., biofilms) are commonly present in natural systems (More et al., 2015).

3.4.2. Dependence of EPS on Growth Phase

The release of enzymes by microbes into their external environment forms the basis for the interaction between cells and high molecular weight exogenous substrates. Chumak et al. (1995) studied complexes formed by EPS and bacteriolytic enzymes produced by *Pseudomonads*. They indicated that great quantities of EPS are excreted into the culture medium along with bacteriolytic enzymes. They found that during growth in batch culture, the ratio of enzyme to EPS varied in different growth phases. The secretion of bacteriolytic enzymes is initiated during the latent growth phase and continues for the duration of culture growth, while the highest rate of EPS production and accumulation occurs during the second half of the exponential growth phase.

Jia et al. (1996) investigated the effects of cultivation time on EPS production in activated sludge. They found the EPS content to be closely related to bacterial growth phase. During the exponential growth phase, EPS content increased with cultivation time, but during the stationary phase it decreased with increasing cultivation time. In contrast, EPS content of a photosynthetic bacterial strain decreased with cultivation time during the exponential growth phase, but remained almost unchanged during the stationary phase (Sheng et al., 2006; Sheng and Yu, 2007).

3.4.3. EPS Extraction Procedures

In pure or mixed cultures the EPS extraction procedure must be selected for each case, considering the specific needs and constraints. In some studies (Seviour et al., 2012), a certain fraction, e.g., the polysaccharides, is extracted for a more detailed chemical or structural analysis. In that case, lysis of the bacteria may not be a problem, if only the impurities can be removed before further analysis (Nielsen and Jahn, 1999).

The best extraction procedure should (i) cause minimal cell lysis, (ii) not disrupt or alter biopolymers, and (iii) release all EPS biopolymers. However, it is hardly possible to meet all of these requirements with the extraction methods known today.

Many chemical extraction methods rely on a breakage of the electrostatic interactions, thereby promoting an extraction of water-soluble compounds. Less focus has been on the hydrophobic components, probably because it is difficult not to destroy the cells with these procedures. It is particularly difficult to test the extraction performance in undefined cultures such as biofilms and flocs. As no universal extraction method exists, it is recommended that extraction is only performed after running some comparative methods and initially optimizing and standardizing a selected extraction technique (Nielsen and Jahn, 1999).

This can be done by changing the variables (extraction time, shear, temperature, or chemical) and by recovery of added standards. Since EPS yield (defined as grams of polysaccharide produced per unit biomass) was shown to depend on extraction time and shear rate (Frolund et al, 1996). Furthermore, in EPS analysis an evaluation of lysis and polymer disruption must be included. A typical procedure of sampling, handling and analysis of bound EPS in bioaggregates is as follows:

a) Sampling and pretreatment: Sampling of microorganisms from the environment or bioreactors. Pretreatment often includes a washing step and homogenization of the sample. The samples might be stored before further handling.
b) Extraction: The EPS components are extracted by an appropriate extraction procedure.

c) Purification: In some cases the extracted EPS are purified before further analysis.
d) Analysis: The EPS are usually analyzed for macromolecular composition (e. g., polysaccharides and proteins). In some cases a more detailed investigation of the chemical composition or other characteristics is performed (sorption, complexation etc.)

3.5. Fractions in EPS

The forms of EPS that exist outside of cells can be subdivided into two as:

- **Bound EPS** (**B-EPS)**
- **Soluble-EPS** (**S-EPS/SMP, soluble microbial products).**

B-EPS are composed of sheaths, **tightly-bound polymers (TB-EPS), loosely bound polymers (LB-EPS),** capsular polymers, condensed gels and attached organic materials. S-EPS are composed of soluble macromolecules, colloids, and slimes (Nielsen and Jahn, 1999;Laspidou and Rittmann, 2002a) (Figure 3.1).

As shown in Figure 3.1, bound-EPS are closely bound with cells, while soluble-EPS are weakly bound or dissolved into the solution. Generally, these two types of EPS can be separated by centrifugation. Those remaining in the supernatant are called S-EPS, while those forming microbial pellets are known as bound EPS. TB-EPS have a certain shape and are stably bound with cell surface. The outer part, which consists of LB-EPS, is a loose and dispersible slime layer without an obvious edge. The content of LB-EPS in microbial aggregates is always less than that of TB-EPS (Sheng et al., 2006;Li and Yang, 2007).

Bacteria convert a fraction of organic substrate into SMP (de Silva and Rittmann, 2000). SMP are a myriad of soluble organic matter produced by microbial populations in bioreactors (Barker and Stuckey, 1999; Laspidou and Rittmann, 2002b). SMP have been found to comprise the majority of soluble organic materials in the effluent of biological treatment systems (Barker and Stuckey, 1999; Aquino and Stuckey, 2004; Rosenberg, 2006). Therefore, their presence is of particular interest in achieving discharge standards for wastewater treatment plants.

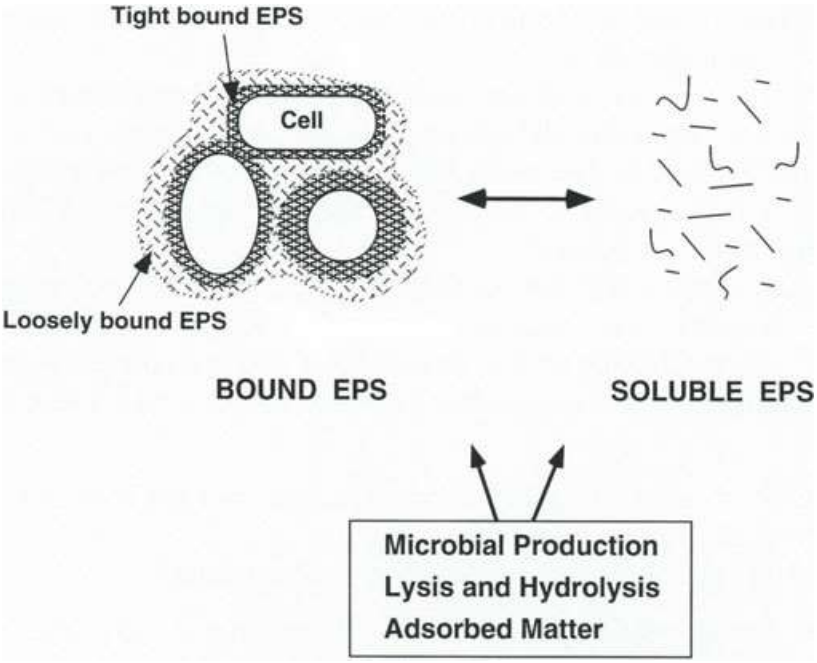


Figure 3.1. Cell biomass and extracellular polymeric substances (EPS) (Wingender et al., 1999).

3.6. Interactions Among Different EPS Fractions

Up to now, some researchers have treated EPS and SMP separately (Costerton et al., 1981; Hsieh et al., 1994; Nielsen et al., 1997). On the other hand, some researchers consider that SMP and EPS are closely related to each other. For example, Laspidou and Rittmann (2002b) propose that cells use electrons from the electron-donor substrate to build active biomass. They also produce bound EPS and utilization-associated products (UAP) at the same time and in proportion to substrate utilization (Figure 3.2). Bound EPS are hydrolyzed to biomass-associated products (BAP), while active biomass undergoes endogenous decay to form residual dead cells. Finally, UAP and BAP, being biodegradable, are utilized by active biomass as recycled electron-donors substrates. Moreover, according to the unified theory, SMP and EPS overlap each other. Soluble EPS is actually SMP, or the sum of UAP and BAP. Furthermore, active biomass includes bound EPS, while inert biomass includes bound EPS and the residual dead cells (Laspidou and Rittmann 2002b).

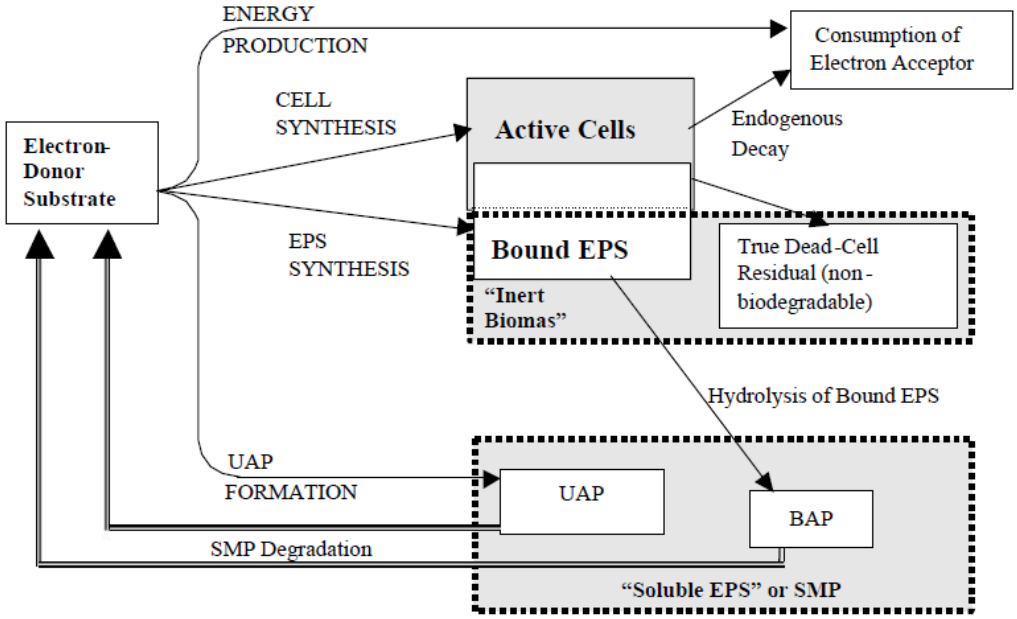


Figure 3.2. Schematic representation of the unified model for active biomass, EPS, SMP, and inert biomass (Laspidou and Rittmann, 2002b).

A common theme of EPS and SMP is that both are microbial products that contain electrons and carbon, but are not active cells (Laspidou and Rittmann, 2002b; Ni et al., 2009). Although SMP are completely soluble, EPS are mostly associated with the solid phase and are therefore insoluble. Ramesh et al. (2006) compared the physicochemical characteristics of SMP and soluble EPS from original and aerobically or anaerobically digested wastewater sludge. In their study, surface charges, particle sizes, and chemical compositions of SMP and soluble EPS containing suspensions were compared. Their experimental results revealed that the particles in SMP and soluble EPS fractions extracted from original wastewater sludge, before and after digestion, were not identical in all physicochemical characteristics herein measured. Thus, they concluded that SMP might not be identical to the soluble EPS from wastewater sludge.

3.7. Characteristics of EPS

3.7.1. Molecular Weight

EPS are of particular importance because these biopolymers are thought to be the glue that holds activated sludge together (Higgins and Novak, 1997; Jorand et al., 1998). In activated sludge, EPS have a broad molecular weight (MW) range between 4800 kDa and <1 kDa. Horan and Eccles (1986) indicated that the MW and composition of EPS are of fundamental importance in determining settling properties of activated sludges. They found that high MW polymers lead to round strong flocs ad improve settling. On the other hand, Gehr and Henry, (1983) and Forster, (1985) stated that poor settling is associated with the presence of a large MW fraction of biopolymers.

EPS macromolecules can be separated on the basis of MW (Higgins and Novak, 1997; Görner et al., 2003; Garnier et al., 2005). Size exclusion chromatography (SEC) or gel-permeating chromatography (GPC) have been successfully used to characterize EPS of activated sludge.

3.7.2. Adsorption Characteristics of EPS

The EPS in microbial aggregates have many sites for adsorption of metals and organic matters, such as aromatics, aliphatics in proteins, and hydrophobic regions in carbohydrates (Flemming and Leis, 2003). This reveals the potential role of EPS in heavy metal sorption to bacterial cells and transport in the environment (Toner et al., 2005; Comte et al., 2008). The binding capability and strength of the bonds between EPS and heavy metals are known to be high, and adsorption obeys Langmuir or Freundlich equations (Bhaskar and Bhosle, 2006; Zhang et al., 2006).

Furthermore, the soluble EPS might have a greater adsorptive ability for heavy metals than the bound EPS (Comte et al., 2006b). Many functional groups in EPS, such as carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups, can complex with heavy metals (Liu and Fang, 2002a; Ha et al., 2010). Based on the estimated numbers of available carboxyl and hydroxyl groups, EPS are regarded to have a very high binding capacity

(Flemming and Leis, 2002; Guibaud et al., 2003, 2006). Proteins, carbohydrates and nucleic acids in EPS all have the abilities to complex with heavy metals (Dignac et al., 1998; Priester et al., 2007).

The binding between EPS and divalent cations, such as Ca^{2+} and Mg^{2+}, is one of the main intermolecular interactions in maintaining the microbial aggregate structure (Mayer et al., 1999). In adsorption of heavy metals onto activated sludge, Ca^{2+} and Mg^{2+} were found to be released into the solution simultaneously, indicating that an ion exchange mechanism was involved (Yuncu et al., 2006).

Negatively charged EPS could bind with positively charged organic pollutants through electrostatic interaction (Esparza-Soto and Westerhoff, 2003). Moreover, proteins in EPS have a high binding strength and capability. The soluble EPS have a higher fraction of proteins than bound EPS. Thus they may have a greater binding capacity than bound EPS (Pan et al., 2010).

3.7.3. Biodegradability of EPS

EPS can also be used by bacteria as sources of carbon and energy. Usually, the main components of EPS are carbohydrates and proteins. In biological wastewater treatment enzymes are responsible for degradation of these polymers. The bacteria in activated sludge can utilize the EPS that are excreted by other bacteria for metabolic activity (Boyd and Chakrabarty, 1994; Zhang and Bishop, 2003). However, Laspidou and Rittmann (2002b) argued that certain parts of EPS cannot be degraded by microorganisms. Wang et al. (2005, 2007) stated that some part of EPS from aerobic granular sludge was biodegradable. EPS present in the outer layer of aerobic granular sludge could not be biodegraded, although those located in the inner layer were biodegradable. The small molecules produced as a result of EPS degradation can be used as carbon and energy sources for cell growth in the case of nutrient shortage. EPS degradation can also result in deflocculation of sludge flocs. The nonbiodegradable portion of EPS may flow with the effluent from reactors and deteriorate the quality of the effluent (More et al., 2014).

3.7.4. Hydrophilicity/Hydrophobicity of EPS

The EPS in microbial aggregates have many charged (e.g., carboxyl, phosphoric, sulfhydryl, phenolic and hydroxyl groups) and apolar groups (e.g., aromatics, aliphatics in proteins, and hydrophobic regions in carbohydrates) (Flemming and Leis, 2003). The formation of hydrophobic areas in EPS would be beneficial for organic pollutant adsorption (Spath et al., 1998). The presence of hydrophilic and hydrophobic groups in EPS molecules indicates that EPS are amphoteric. The relative ratio of these two groups is related to the composition of EPS.

3.8. Key Factors Affecting the Formation of EPS

3.8.1. Solids Retention Time (SRT)

SRT has a considerable effect on the production of EPS, but the results reported in literature are somewhat contradictory. Many researchers have found that EPS in activated sludge systems increase with an increasing SRT, which implies that bacteria produce more EPS under endogenous conditions. Sesay et al. (2006) found that an increase in SRT had a significant and positive correlation with the total quantity of EPS in activated sludge as well as the contents of proteins and carbohydrates in EPS. The ratio of proteins to carbohydrates also increased from 1.5 to 2.5 with an increase in SRT from 4 to 20 days. However, some researchers have suggested that EPS are independent of SRT. For example, Liao et al. (2001) found that the EPS of activated sludge content did not change significantly with a longer SRT. However, the protein/carbohydrate ratio was found to increase as the SRT increased from 4 to 12 days, but remained unchanged as the SRT increased from 12 to 16 days. Li and Yang (2007) reported that TB-EPS of activated sludge had no relationship with SRT, but LB-EPS decreased with an increasing SRT.

3.8.2. The Type of Substrate in a Wastewater

Substrate type has a substantial effect on microbial metabolism and community. Thus it influences also the production of EPS. For example, Li and Yang (2007) reported that the sludge fed with glucose had more EPS production than that fed with acetate.

Sponza (2002, 2003) examined the EPS production in sludges from continuously stirred tank reactors treating various types of wastewaters. They found that under steady-state conditions the protein content was higher in the EPS of sludge treating winery and municipal wastewaters compared to sludge treating pulp and paper, textile and petrochemical wastewaters.

3.8.3. Nutrient Content

EPS can be degraded by bacteria as carbon and energy sources when there is a substrate shortage. Nutrient levels have a significant effect on EPS production and composition. The EPS content of sludge increased with an increase in food to microorganism ratio (Janga et al., 2007). EPS production could be promoted when phosphorus was in short supply (Liu et al., 2006). Hoa et al. (2003) also found that the carbohydrate content in EPS extracted from activated sludge increased when phosphorus was in short supply. Durmaz and Sanin (2001) found EPS in activated sludge to be rich in proteins but low in carbohydrates at a C/N ratio of 5. As the C/N ratio was increased first to 17.5 and then to 40, carbohydrate concentration increased sharply and protein concentration decreased. Other researchers have found that activated sludge growing on a wastewater with a low C/N ratio tended to produce EPS with a high protein/carbohydrate ratio (Liu and Fang, 2003).

3.8.4. External Conditions

As EPS are bound with cells mainly through ion bridging with multivalent metals, metal concentration may also influence the EPS content. Turakhia and Characklis (1989) and Sheng et al. (2006) reported that the Ca^{2+} concentration had no effect on EPS, whereas Higgins and Novak (1997) found that the protein content in EPS increased at higher Ca^{2+} or Mg^{2+} concentrations, and that higher Na concentrations led to a lower protein content.

In the presence of toxic substances such as heavy metals, microbial cells in activated sludge and biofilms produced more EPS to protect themselves against the harsh environment (Fang et al., 2002; Aquino and Stuckey, 2004). These toxic substances greatly influenced the ratio of proteins to carbohydrates (P/C) in EPS. Sheng et al. (2005)

investigated the production of EPS in the presence of different heavy metals by using a hydrogen-producing photosynthetic bacteria strain, *Rhodopseudomonas acidophila*. Results showed that the P/C ratio in EPS was always higher in the presence of toxic substances than the control. Furthermore, under toxic conditions, the increase in the protein content far exceeded than others, suggesting that extracellular proteins could protect cells against toxic substances.

Also, the shear rate in reactors has an influence on composition of EPS. Microorganisms in high shear environments adhere to surfaces by secreting EPS to resist damage of suspended cells by environmental forces. For example, an increase in shear rate or aeration intensity in a Sequencing Batch Reactor (SBR) could increase the EPS content in sludge (Adav et al., 2007). Also the carbohydrate content of EPS extracted from activated sludge increased with increasing air flow rate in an SBR, whereas the protein content remained almost unchanged at various air flow rates. Shin et al. (2000) indicated that shear may stimulate bacteria to produce more carbohydrates.

In addition, also aerobic or anaerobic conditions can have an influence on the production of EPS. Shin et al. (2000) compared the EPS production of activated sludge in three bioreactors and found that at a high dissolved oxygen level, the production of carbohydrates in EPS increased with time, whereas the protein content remained unchanged. At a low dissolved oxygen level, the concentrations of both carbohydrates and proteins were kept at the same level. The EPS content of sludge would decrease under anaerobic conditions (Mikkelsen and Nielsen, 2001). It is reported that activated sludge flocs tend to disintegrate under oxygen limitation conditions. Such a disintegration might be caused by suppression of EPS production or hydrolysis of EPS.

3.9. Role of EPS on Sludge and Effluent Quality

The interactions between EPS and cells have a significant effect on microbial flocculation ability (Morgan et al., 1990). In a study, bacteria and EPS were found to make up the main part of deflocculated matter (Wilén et al., 2000), which indicated that EPS play an important role in flocculation.

Many studies have demonstrated that EPS have a negative effect on the settleability of activated sludge (Liao et al., 2001; Jin et al., 2003; Ni, 2012). As EPS are negatively charged, a high concentration of EPS increases the surface charge of microbes, which results in an increase in the repulsive forces between cells and a decrease in settleability of activated sludge (Morgan et al., 1990). EPS can also be regarded as a key factor in the thickening and dewatering of sludge (Houghton et al., 2001; Mikkelsen, 2002).

The formation of granular sludge is influenced by complex interactions between EPS and microbial cells. Sludge granulation refers to the self-immobilization of microbes in biological wastewater treatment reactors, which results in a compact structure of aerobic and anaerobic granules. There are plenty of EPS in the interior of aerobic and anaerobic granules (de Beer et al., 1996; Yu et al., 2001; Mcswain et al., 2005). A study indicated that aerobic and anaerobic granules contained more EPS than flocs (de Beer et al., 1996; Zhu et al., 2015). Another study showed that carbohydrate content in EPS and granule strength decreased simultaneously, suggesting that EPS played a crucial role in sludge granulation and maintenance of structure (Quarmby and Forster, 1995).

SMP have been found to comprise the majority of soluble organic material in the effluents from biological treatment systems (Barker and Stuckey, 1999; Aquino, 2004; Rosenberger et al., 2006). They are of crucial importance in activated sludge systems because of their significant impacts on effluent quality and treatment efficiency (Barker and Stuckey, 1999; Jarusutthirak and Amy, 2007). In addition to contributing to the effluent organic level, SMP can have further implications on process performance, although the effect of high concentrations of these products is of interest (Chudoba, 1985, Laspidou and Rittman, 2002a, 2002b).

Chudoba (1985) concluded from his studies that microbial products in high concentrations adversely affected the kinetic activity and the flocculating and settling properties of activated sludge. Among these microbial waste products, EPS, although strictly not soluble, are believed to have the major influence on the settling and flocculating properties of activated sludge, as mentioned previously (Barker and Stuckey, 1999). Indeed, they are now manufactured artificially to be used as alternatives to other

synthetic or natural water-soluble polymers or as novel polymers in thickening, suspending, and gelling applications (Barker and Stuckey, 1999).

3.10. Heavy Metals

The term heavy metal refers to any metallic chemical element that has a relatively high density and is toxic or poisonous at low concentrations. Heavy metals are natural components of the Earth's crust. They cannot be degraded or destroyed. To a small extent they enter our bodies via food, drinking water and air. As trace elements, some heavy metals (e.g. copper, selenium, zinc) are essential to maintain the metabolism of the human body. However, at higher concentrations they can lead to poisoning. Heavy metal poisoning could result, for instance, from drinking-water contamination (e.g. lead pipes), high ambient air concentrations near emission sources, or intake via the food chain (Merrill et al., 2007).

Human’s use of metals seriously began to affect the environment during the Industrial Revolution. From the environmental point of view, metals are of greatest concern due to their presence or accumulation. They can have a toxic or inhibitory effect on living things (Forster and Wase, 2003). Metals can be dispersed, both naturally and by man’s activities, into any of the earth’s elements: soil, water or air. The metals which are of environmental concern are listed in Table 3.1. The main heavy metals mentioned in environmental sciences are lead, mercury, cadmium, chromium, copper, manganese, nickel, zinc and silver.

Table 3.1. Some sources of heavy metals.

METALS	SOURCES
Lead	Storage batteries, insecticides, plastic, water pipes, food and beverages medicinal concoctions.
Cadmium	Paint pigments, electroplating, plastics, silver cadmium batteries, phosphate fertilizer.
Mercury	Pesticides, fertilizer, pulp and paper, high intensity street lamps Thermometers.
Silver	Circuit boards, photographic prints, dental fillings, old black and white films,nonrecycled coins.
Zinc	Wood preservatives, ceramics, photographic paper, textiles, fertilizers, batteries, steel production.
Nickel	Silver refineries, electroplating, Zinc base casting, storage battery industries.
Copper	Mining, fertilizers, printed circuits, pulp and paper, fungicidal sprays and animal wastes.

3.10.1. Heavy Metals in Biological Treatment Systems

The metals found in treatment systems typically include copper, cobalt, arsenic, mercury, aluminum, gold, tin, lead, zinc, nickel, silver, cadmium, chromium etc. and originate predominantly from industrial discharges. Stringent effluent regulations and concerns about the receiving environment and biota have resulted in a need to assess the interaction and removal efficiency of heavy metals in biological treatment systems (Cheng et al., 1975; Savvides et al., 1995).

Physicochemical methods such as chemical precipitation, membrane filtration, ion exchange, flotation, reverse osmosis, membrane filtration, solvent extraction and activated carbon adsorption have been developed for removal of heavy metals from wastewater. However, the practical application of such processes is sometimes restricted due to technical or economical constraints. The biological removal of metals through biosorption

has distinct advantages over conventional methods: the process rarely produces undesirable or deleterious chemical byproducts and it is highly selective, efficient, easy to operate, and cost effective in the treatment of large volumes of wastewater containing toxic heavy metals (Mullen et al., 1989; Volesky, 1990). Microorganisms can remove heavy metals from wastewaters by binding the cationic metals onto negatively charged functional groups distributed on their cell walls, such as carboxyl and phosphoryl groups. For example, literature findings showed that biosorption mechanisms were dependent on the functional groups on aerobic granules (Gao et al., 2011). The cell surfaces of Gram-positive and Gram-negative bacteria, whether living or nonliving, possess abundant functional groups that bind metal ions. These also include phosphate, carboxyl, hydroxyl, and amino functional groups, among others (Wingender et al., 1999).

According to the location where metal biosorption occurs, the mechanisms of biosorption are classified as follows: Intracellular interaction, cell surface interaction or extracellular interaction (Kim and Kang, 2006).

Active transport of the metal across the cell membrane leads to intracellular accumulation, which depends on bacterial metabolism (Veglio and Beolchini, 1997). Essential metals are actively taken up by specialized uptake systems because they are needed by bacteria, but other, nonessential metals may also be taken up because they are mistaken for an essential metal (Ledin, 2000). When microorganisms are exposed to high concentration of toxic metals, they actively take up metal ions to detoxify the surrounding environment. Actually, a variety of bacteria is capable of converting metal and metalloid ions to organometallic and organometalloid compounds by intracellular ligands such as metallothioneins. The microorganism has also a metabolically sponsored process such as bioprecipitation to enhance the metal uptake (Valls and Delorenzo, 2002). In that case, the soluble metal ion is converted into an insoluble compound and precipitates out.

The toxic level of metals can change depending on the type of organism such as plant, microorganism and algea. For instance, a group of researchers applied ten different heavy metals and biomonitored the behaviour of the plant *Lemna minor*, clone St (Naumann et al., 2007). The growth inhibition was quantitatively measured (effective dose required for the inhibition of growth rates by 50%, EC_{50}). Based on the averages of all

tested parameters, the following phytotoxicity series was obtained: $Ag^{+} > Cd^{2+} > Hg^{2+} > T^{1+} > Cu^{2+} > Ni^{2+} > Zn^{2+} > Co^{6+} > Cr^{6+} > As^{3+} > As^{5+}$ (Appenroth, 2010).

Çeçen et al. (2010a, b) investigated the inhibitory effects of eleven different metals on a nitrifying sludge based on the assessment of the IC_{50} concentrations leading to 50% inhibition. The method based on respiration of nitrifying sludge (oxygen uptake rate, OUR) in the presence of these metals. Calculated IC_{50} values showed that the order of inhibitory effect was as $Ag^{+} > Hg^{2+} > Ni^{2+} > Cd^{2+} > Zn^{2+} > Cu^{2+} > Co^{2+} > Cr^{3+} > Cr^{6+}$ in the nitrification system. Since Pb precipitated out, IC_{50} values could not be calculated. Moreover, lithium was not toxic to nitrifiers even at high concentrations.

Another study showed that copper possessed the highest toxicity towards biomass in the order $Cu^{2+} > Cd^{2+} > Cr^{6+} > Co^{2+}$, as determined by IC_{50} values (El Bestawy et al., 2013).

3.10.2. Silver

The major sources of silver in the environment are wastes from electronics, jewelry, soldering, bearings, and for medical and dental applications (Wang et al., 2003). Survey results indicated that about 2.47 million kg of silver are lost each year to the domestic biosphere, mostly as a result of human activities. The photography industry accounts for about 47% of silver discharged into the environment from anthropogenic sources. An estimated 150,000 kg of silver enter the aquatic environment every year from the photography industry, mine tailings, and electroplaters (Irwin et al., 1998).

The silver ion is known to complex or precipitate with many organic and inorganic materials such as chloride, sulfide, thiosulfate, and dissolved organic carbon (Ratte, 1999). For example, the solubility products of silver chloride and silver sulfide (K_{sp}) are, respectively, $10^{-9.75}$ and $10^{-48.97}$ (Benjamin, 2002), indicating that the free Ag^{+} concentration is extremely low if chloride and/or sulfide are present (Lytle, 1984).

As indicated above, some heavy metals such as Cu^{2+}, Ni^{2+}, Co^{2+} and Ag^{+} can form complexes with dissolved organic matter (DOM) and EPS (Rudd et al., 1982; Ratte, 1999;

Guibaud et al., 2004; Comte et al., 2008). However, little information has been reported regarding the reactions between Ag^+ and EPS.

3.10.3. Silver Nanoparticles (AgNP)

Today, nanoscale materials find use in a variety of different areas such as electronic, biomedical, pharmaceutical, cosmetic, energy, environmental, catalytic and material applications. Because of the potential of this technology there has been a worldwide increase in investment in nanotechnology research and development (Guzmán et al., 2006). The benefits of nanoparticles, however, need to be weighed with any potential cost to the environment and public health. The unknown health effects and risks associated with these materials have drawn considerable attention from researchers, consumers and regulators (El-Badawy et al., 2010).

Silver nanoparticles (AgNPs, nanosilver) have acquired a variety of applications in consumer products because of their antimicrobial properties (Markarian, 2006; Kim et al., 2007). With expanding use of nanosilver products, the release of nanosilver particles into sewage collection systems and WWTPs becomes a growing concern. Nanoparticles are able to attach to cell membranes, resulting in changes of membrane permeability and redox cycling in the cytosol, accumulation of intracellular radicals, and dissipation of the proton motive force for ATP synthesis. Each of these has been reported as a possible mechanism of nanosilver toxicity (Sondi and Salopek-Sondi, 2004; Morones et al., 2005; Nel et al., 2006). Smaller particles (<10 nm) may enter the bacterial cell directly to cause further damage by interfering with DNA and protein synthesis (Morones et al., 2005).

Nanosilver inhibits bacterial growth (Sondi and Salopek-Sondi, 2004; Panáček et al., 2006). For instance, autotrophic nitrifying bacterial growth was inhibited by about 80% at 1 mg/L of silver nanoparticles (average size 14±6 nm) (Choi et al., 2008). During the course of this thesis, silver (Ag^+) and nanosilver (AgNP) were selected as the two heavy metal forms. In view of literature findings, these species can bind onto biomass and complex with EPS. The possible binding mechanisms are shown in Figure 3.3.

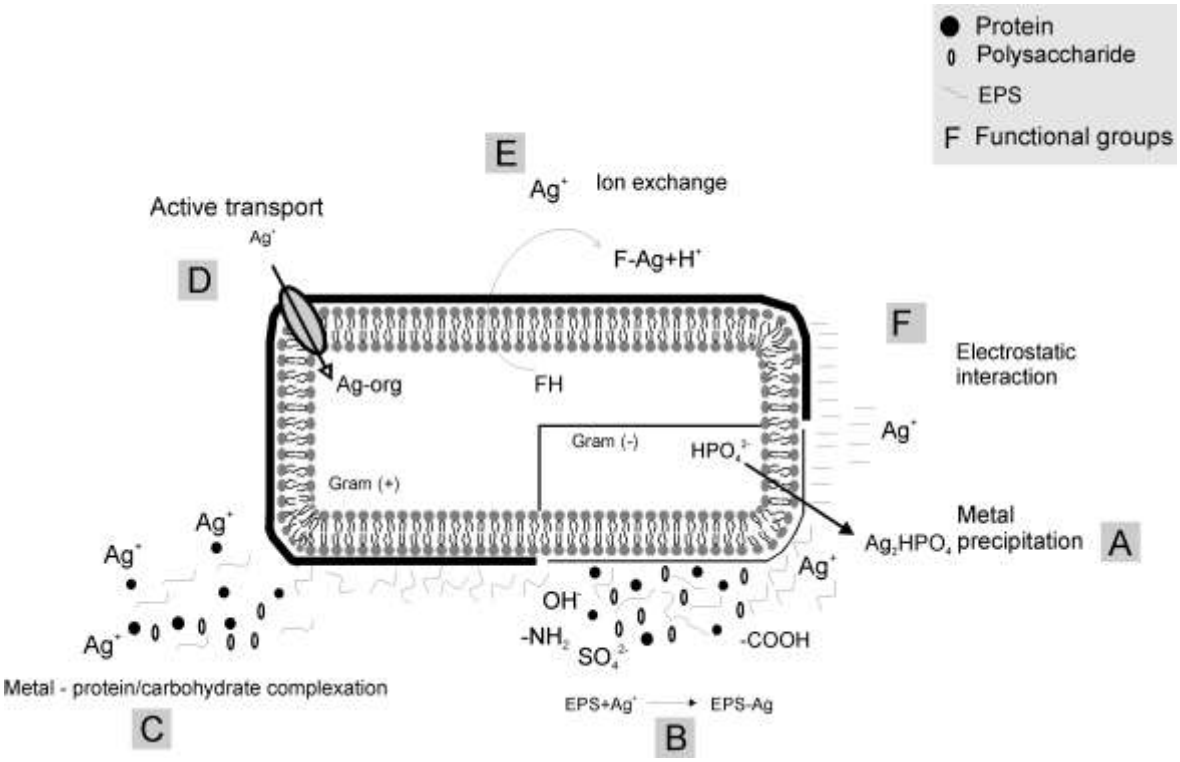


Figure 3.3. Possible mechanisms for sorption of Ag species onto bacteria.

3.10.4. Surface Properties of AgNP and Bacterial Inhibition

Bare AgNPs have a negative surface charge throughout the pH range common in the environment (3 to 9); however, this surface charge can be modified from approximately +30 mV to −60 mV using organic coatings. This variable surface charge may play a large role in bacterial growth inhibition. The positively charged branched polyethylenimine (BPEI) coated AgNPs were found to be the most effective inhibitors of Gram positive bacterial growth (Levard et al., 2012). The researchers hypothesized that attachment of AgNPs to the negatively charged bacterial cell walls is the cause of this charge dependent toxic effect. Other types of NPs such as gold nanorods have demonstrated a similar toxic effect due to positively charged coatings (Leonov et al., 2008; Alvarez-Puebla and Aroca, 2009).

NPs can be stabilized by a large variety of stabilizers. Citrate is the most ubiquitous carboxylic acid used as a capping (and reducing) agent (Allen et al., 2010; Tolaymat et al., 2010; Croteau et al., 2011; Huynh and Chen, 2011), but carboxylic acids with alkyl chains (e.g., oleic acid) are also used (Shoults-Wilson et al., 2011). Various types of polymers

have been used as coatings including polyvinylpyrrolidone (Tolaymat et al., 2010; Huynh and Chen, 2011; Levard et al., 2011), polyacrylate (Stebounova et al., 2011), poly(vinylalcohol) (Asharani et al., 2010), polyacrylamide (Morones and Frey, 2007) and thiol-modified oligonucleotides (Vidal et al., 2005). Polysaccharides are common coatings including compounds like gum Arabic, sophorolipids and other sugars (Song et al., 2011). Biological macromolecules such as bovine serum albumin (BSA) (Asharani et al., 2008) and fatty acids (Tolaymat et al., 2010) have also been used to stabilize AgNPs against aggregation.

Hydrophobic groups in the organic coating are an additional factor that must be considered (Levard et al., 2012). Dutta et al. (2011) studied the effects of different amphiphile coatings on the surface of AgNPs. They found that the coating containing a hydrophobic group had a 5-fold lower minimum inhibitory concentration (i.e., higher toxicity) for *B.subtilis* bacteria than AgNP coatings without hydrophobic groups. Although this effect was not demonstrated for other bacteria (*Micrococcus luteus* and *Staphylococcus aureus*) or fungi (*Candida albicans* and *Saccharomyces cerevisiae*), this finding is consistent with another study. The study showed that the relative hydrophobicity of polymeric coatings on AgNPs affects their attachment to hydrophobic surfaces, with greater attachment for more hydrophobic coatings. It is clear from these few studies that surface charge and organic coating type play a role in inhibition of bacteria (Dutta et al., 2011; Panacek et al., 2011).

Although a positive nanoparticle surface charge has a clear inhibitory effect on bacterial growth in some systems, in other cases there is no clear effect caused by altering the surface charge from positive to negative. For example, Dror-Ehre et al. (2009) synthesized two types of AgNPs capped with 3-mercaptopropionic acid as an anionic stabilizing agent (−46.1 mV zeta potential) and polylysine as a cationic stabilizing agent (40.2 mV zeta potential). In both cases, regardless of surface charge, a 5 orders of magnitude reduction was observed in bacterial viability. The researchers suggested that further investigation is needed to characterize the surface characterization of AgNP and to elucidate why bacterial growth is nonsensitive to surface charge.

Another key component altering the surface charge of AgNPs in environmental systems is the Natural Organic Matter (NOM). Nanoparticles of any charge, especially positive, interact with and likely adsorb parts of these macromolecules. For example Fabrega et al. (2009) found that Suwannee River humic acid (a specific type of NOM) at 10 mg/L caused the AgNP surface to be more negatively charged than bare AgNP, somewhat decreasing inhibitory effects of AgNP on bacterial growth. Additionally, NOM plays a role in complexing Ag^{+}, but these interactions have yet to be fully determined (Levard et al., 2012).

3.10.5. Effect of Nanoparticle Aggregation on Toxicity

There is a growing evidence that small nanoparticles (i.e. < 20− 30 nm in diameter) can display properties significantly different relative to larger nanoparticles or bulk materials of the same composition. It is also clear that nanoparticle surface area is predictably related to the amount of free ion released into suspension. This relationship between surface area and ion release also tranfers well to toxicity studies, where smaller sized particles have exhibited larger toxic responses (Levard et al., 2012). However, in many past studies, size and state of aggregation were often measured before experiments only. Such studies did not determine if nanoparticle size and/or aggregation state changed during experiments. One of these studies (Römer et al., 2011) related the aggregation state of citrate-stabilized AgNPs to its toxic effect on *Daphnia* and concluded that the medium needs to be diluted at least 10-fold to reduce aggregation effects significantly.

Nanoparticle aggregation will likely have an effect on toxicity by reducing the rate of dissolution, uptake by organisms, and stability of the nanoparticles against aggregation. Supporting this suggestion is a recent study by Reinsch et al. (2012) which showed that aggregated AgNPs have a greater inhibitory effect on the growth of *E. coli* than dispersed AgNPs, possibly due to lower sulfidation of AgNPs in aggregates.

3.10.6. Fate of AgNPs in Wastewater Treatment Plants

Due to extensive usage of NPs, the production of engineered nanomaterials was expected to increase to 58,000 tons in 2011-2020 (Maynard, 2006). Models predicting the

environmental loads of AgNPs suggest that nanoparticles released from domestic and industrial sources will in most cases enter sewer systems and eventually WWTPs. For example, nanoparticles can leach out of clothing in just a few washes (Benn and Westerhoff, 2008) and thus enter WWTPs. However, little is known about the fate of AgNPs in WWTPs.

Environmental transformations of AgNPs need to be investigated to determine the change in their surface properties and reactivity (e.g., dissolution), which will, in turn, affect their transport, reactivity, and toxicity in soils and natural waters. Strangely, very few studies have focused on the environmental transformations of AgNPs. Most commonly, Ag^+ strongly reacts with reduced sulfur ligands to form a silver sulfide (Ag_2S) in wastewaters. From an environmental perspective, it is crucial to study sulfidation processes since the majority of AgNPs may end up in sewage pipes and finally in wastewater treatment plant effluent and biosolids. This transformation is thermodynamically expected considering the pH and the redox conditions in the settling tanks, especially in light of the high concentrations of sulfide in these types of facilities compared to natural waters (Levard et al., 2012).

Some researchers discovered the presence of Ag_2S nanoparticles in sewage sludge products (Kim et al., 2010; Kaegi et al., 2011). Although the source of Ag is uncertain, authors hypothesize that because naturally occurring crystals of Ag_2S are rare, Ag_2S nanoparticles are formed during wastewater treatment by the reaction of either AgNPs or soluble Ag ions with reduced sulfur species (Levard et al., 2012). Ag_2S-NPs were identified in the final stage sewage sludge materials of a full-scale municipal wastewater treatment plant (Kim et al., 2010). More recently, sulfidation of AgNPs was demonstrated in a pilot plant fed with municipal wastewater and spiked with AgNPs (Kaegi et al., 2011).

TEM analysis, X-ray absorption spectroscopy, and compositional analysis of the sludge showed that 85% of Ag was sorbed to wastewater biosolids as Ag_2S, whereas only 5% of Ag was found in the effluent after 43 days (Kaegi et al., 2011). Silver in the effluent was also sulfidized to form Ag_2S. AgNPs will most likely be transformed into Ag_2S before entering WWTPs due to the relatively high (up to 6 mg/L) sulfide concentrations in sewer pipes (Nielsen et al., 2008). Human and industrial activities increase the generation of

corrosive gas dissolved in water, including H_2S (Wiener et al., 2006). Furthermore, ppm levels of dissolved H_2S have also been reported in rivers, seas, and brackish polluted aerobic waters (Wiener and Salas, 2005). In summary, the majority of AgNPs released into wastewater will most likely be transformed into Ag_2S and incorporated into sewage sludge. Then they may reenter the environment via application of sewage sludge to agricultural lands or from disposal of incinerator residues.The rate of sulfidation under environmentally relevant conditions should be determined as well as the stability of AgS_2 against oxidation of sulfur and subsequent release of Ag ions (Levard et al., 2012).

WWTPs have proven to be very efficient at removing silver from treated water (Levard et al., 2012). For example, in one literature work it was found that, for influent Ag concentrations up to 1.85 mg/L, Ag could be treated and had no effect on the performance of aerobic wastewater biodegradation (Pavlostathis and Maeng, 1998). Kaegi et al. (2011) stated that most of Ag was found to be associated with sludge solids.

According to the results of our previous works (Çeçen et al., 2010a, 2010b) on the inhibitory concentration of different heavy metals (Ni, Pb, Cu, Co, Cr, Ag, Zn, Hg,) and alkali metal (Li) in a nitrification system, Ag proved to be the most toxic metal among all. It had an 21 h-IC_{50} value of 0.33 mg/L when O_2 consumption was considered. The toxic effect of this metal was one to two orders of magnitude higher than Cd, Pb, Hg and Cr. A high inhibitory effect was observed at already very low concentrations of Ag^+. According to theoretical calculations, after contact with biomass, most of Ag^+ was bound to biomass. Most probably, it was the free form of Ag^+ that was directly taken onto/into biomass causing toxicity, the key species emerged therefore as the free and biomass-bound Ag. In all runs Ag exerted its toxicity immediately upon contact with biomass.

3.11. Determination of Metals by Voltammetry

Polarography or voltammetry (VA) are the names of analytical methods based on current-potential measurements in electrochemical cells. The analytical signal is the current – normally a faradaic current – which flows through the cell during the reaction of the analyte at the working electrode with a small surface. The analyte may be a cation, an anion or a molecule (Henze, 2003). The founder of this method, Jaroslav Heyrovský,

introduced the dropping mercury electrode (DME) as the working electrode. The electrode consists of a thick-walled glass capillary from which the mercury drops into the sample solution under the pressure of a mercury column. In his paper “Electrolysis with the dropping mercury cathode” (1923), he called the recorded current-potential curves polarograms and introduced the term polarography (Heyrovsky, 1923).

Analytical chemists routinely use voltammetric techniques for the quantitative determination of a variety of dissolved inorganic and organic substances. Inorganic, physical, and biological chemists widely use voltammetric techniques for a variety of purposes, including fundamental studies of oxidation and reduction processes in various media, adsorption processes on surfaces, electron transfer and reaction mechanisms, kinetics of electron transfer processes and transport, speciation and thermodynamic properties of solvated species. Voltammetric methods are also applied to the determination of compounds of pharmaceutical interest and, when coupled with HPLC, they are effective tools for the analysis of complex mixtures (Kounaves, 1997).

The electrochemical cell, where the voltammetric experiment is carried out, consists of a working (indicator) electrode, a reference electrode, and usually a counter (auxiliary) electrode (Figure 3.4). In general, an electrode provides the interface across which a charge can be transferred or its effects felt. The reduction or oxidation of a substance at the surface of a working electrode, at the appropriate applied potential, results in the mass transport of new material to the electrode surface and the generation of a current. Even though the various types of voltammetric techniques may appear to be very different at first glance, their fundamental principles and applications derive from the same electrochemical theory.

According to the rules of International Union of Pure and Applied Chemistry (IUPAC), the term polarography should always be used when the current-potential curve is recorded by using a liquid working electrode whose surface can be renewed periodically or continuously (e.g. by drops). This includes the classical DME and the subsequently developed static mercury drop electrode (SMDE). Moreover, the hanging mercury drop electrode (HMDE), the thin mercury film electrode (TMFE), glassy carbon electrodes (GCE) and carbon paste electrodes (CPE) are other possible electrode types (Henze, 2003).

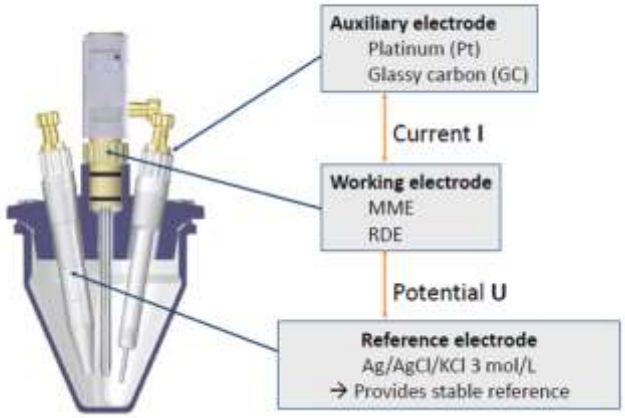


Figure 3.4. Schematic representation of the three electrode system.

There are different available voltammetric techniques which are capable of measuring very small quantities of free and labile metal ions in solution:

- Pulse Polarography (PP)
- Differential pulse polagragphy (DPP)
- Anodic stripping voltammetry (ASV)
- Differential pulse anodic stripping voltammetry (DPASV)

For concentrations in the range of 10^{-7} to 10^{-4} mol/L the test solution is usually analysed directly using DPP and a DME or fast linear scan voltammetry with mercury or solid electrodes. For lower concentrations, stripping techniques following electrolytic or adsorptive accumulation at the electrode (usually mercury) are used (Henze, 2003). Accuracy varies with technique from 1 to 10%.

3.11.1. Use of Voltammetric Technique in Environmental Samples

The effluents contaminated with heavy metals and some portions of metals will pass through the treatment plant to be discharged to surface waters. Heavy metals have a negative influence on the efficiency of biological treatment. Knowledge of their total concentration in wastewater samples is usually not sufficient to determine their effects. It is necessary to assess their speciation, namely to examine the distribution of metal among

different chemical forms (Florence, 1982). Accordingly, the efficiency with which a sewage treatment plant retains influent metals will depend upon the physicochemical forms of metals and their behaviour in physical and biological treatment processes.

Speciation measurements have been made by using various techniques (Florence et al., 1980). Generally, metal speciation and distribution between the soluble and particulate (bacterial solid) phases were calculated using soluble ligand data, equilibrium constants available in literature (Smith et al., 2015). On the other hand, total metal concentration in wastewaters can be measured by using Atomic Absorption Spectroscopy (AAS) and Inductively Coupled Plasma (ICP). VA provides information on speciation of heavy metal traces in natural waters or pharmaceutical industries (Florence et al., 1980; Buffle and Tercier-Waeber, 2005; Farghaly et al., 2014). However, voltammetric applications in wastewater have been limited because of complex composition of wastewaters.

In literature only a limited number of studies is available for the determination of complexation of metals with EPS. For example, Comte et al. (2006) found that EPS had a greater affinity for Pb^{2+} than for Cd^{2+}. Another study provided information on the metal complexation potential of EPS (Guibaud et al., 2005). EPS were extracted from activated sludges. Results showed that, EPS exhibited a greater ability to complex with Pb than Ni. Overall, Cd showed the weakest affinity (Guibaud et al., 2005). Moreover, Semerci and Çeçen (2007) investigated the influence of Cd speciation on nitrification inhibition in batch suspended growth activated sludge systems enriched in nitrifiers. Free (Cd^{2+}), labile and biosorbed Cd were measured by using voltammetry. They found that a quite high Cd level may not lead to inhibition of nitrification due to the complexing potential of Cd with inorganic and organic ligands. This leads to the result that free and labile metal concentrations should also be considered in inhibition.

The complexation between EPS and AgNP is important regarding effluent discharge and sludge disposal. For example, AgNP can be released into aquatic environment due to the complexation with soluble and loosely bound-EPS fractions. On the other hand, AgNP can be released to rural areas since it complexes with tightly bound-EPS (attached to microbial cells). In literature, such a complexation study is not available.

4. MAIN STEPS OF THE STUDY

This study was mainly conducted within the scope of a TÜBİTAK project (Project No. ÇAYDAG-111Y018, Microbial products and metal inhibition in biological systems) which investigated the inhibitory effect of silver metal on different activated sludges (Ayyıldız, 2013; Kılıç, 2014) as well as the relationship between inhibition and EPS characteristics (this dissertation). Table 4.1 shows the main steps in this dissertation.

Table 4.1. Methodology of this study.

SCFB Reactor Operation	**Part I: Effect of COD/TKN ratio on EPS production**	**Part II: Effect of organic carbon type on EPS production**	**Part III: Effect of Ag^+ and AgNP on EPS production**	
1. Sludge Maintenance & Growth			1. Short-term experiments	2. Long-term experiments
• Start up of the main reactor. • Start up of R1, R2, R3 reactors having COD/TKN ratios of 10, 5, 0 (Part I). • Start up of CR, RG, RP reactors feeding with different organic carbon source at constant COD/TKN ratio (10) (Part II). • Start up of CR_{AgNP}, RP_{AgNP} reactors receiving AgNP with feed (Part III).	• Reactors: R1, R2 and R3 • EPS extractions • EPS analysis • MW determination of EPS • Data evaluation	• Reactors: CR, RG and RP • EPS extractions • EPS analysis • MW determination of EPS • Surface characteristics • Data evaluation	• Reactor: CR • Respirometric experiments • EPS extractions • EPS analysis • MW determination of EPS • Voltammetric experiments • Data evaluation	• Reactors: CR_{AgNP}, RP_{AgNP} • Respirometric experiments • EPS extractions • EPS analysis • MW determination of EPS • Surface characteristics • Data evaluation
2. Analytical Methods COD, MLSS, MLVSS				

5. MATERIAL AND METHODS

5.1. Reactor Operation

The activated sludge used in experiments was initially taken from the recycle line of the municipal wastewater treatment plant Paşaköy in Istanbul on April 2012. The mother reactor was fed with synthetic wastewater (Feed 1) as shown in Table 5.1. The reactor was aerated both with air pumps and using the compressed air line. The reactor was operated in the semi-continuous fed batch (SCFB) mode. When the reactor reached steady-state conditions with respect to MLSS and MLVSS, the sludge was divided into different reactors. Reactors were fed with synthetic wastewaters called as "feeds". Each feed had a particular COD/TKN characteristic (Table 5.1). The details of reactor operation can be found elsewhere (Ayyıldız, 2013; Kılıç, 2014).

Table 5.1. Composition of concentrated feeds used in Part I and Part II.

	Part I			**Part II**		
	Different C/N ratio			**Same C/N ratio, different organic carbon source**		
	R1 Feed 1 (C/N=10)	**R2 Feed 2 (C/N=5)**	**R3 Feed 3 (C/N=0)**	**CR Feed 1 (C/N=10)**	**RP Feed P (C/N=10)**	**RG Feed G (C/N=10)**
Constituents	**Concentration (mg/L)**			**Concentration (mg/L)**		
$C_6H_{12}O_6$	5600	2800	0	5600	0	9370.4
$CH_3COONa \cdot 3H_2O$	8000	4000	0	8000	0	0
peptone water	2000	1000	0	2000	16051	0
$(NH_4)_2SO_4$	4000	4360	4714	4000	283	4714
$NaHCO_3$	12000*	12000	12000	2250	4500	4500
K_2HPO_4	1000	1000	1000	1000	1000	1000
KH_2PO_4	1000	1000	1000	1000	1000	1000
$MgSO_4$	1000	1000	1000	1000	1000	1000
$MnSO_4.H_2O$	25	25	25	25	25	25
$CaSO_4$	500	500	500	500	500	500
$FeSO_4.7H_2O$	343	343	343	343	343	343

*In later periods of operation, $NaHCO_3$ was lowered to 2250 mg/Lin stock feed.

In relation to this thesis, eight different bench-top activated sludge reactors were operated over 450 days (Ayyıldız, 2013; Kılıç, 2014). The long-term semi-continuous feeding at steady loading led to a steady effluent concentration. Thus, it may be assumed that this type of reactor operation resembled a CSTR operation at steady-state. Daily 1/20 of sludge was wasted from reactors to keep a sludge age of 20 days.

5.1.1. Part I: Reactors Operated at Different Carbon to Nitrogen (COD/TKN) Ratios

In the first part of reactor operation, reactors were operated semi-continuously at different C/N (COD/TKN) ratios as 10, 5 and 0. For this purpose, three different reactors were used, namely R1, R2 and R3, each representing a different activated sludge system. The configuration of these reactors is shown in Figure 5.1. They were constantly aerated with air diffusers and kept at 25 °C using temperature probes. Dissolved oxygen was kept at about 6.5 mg/L.

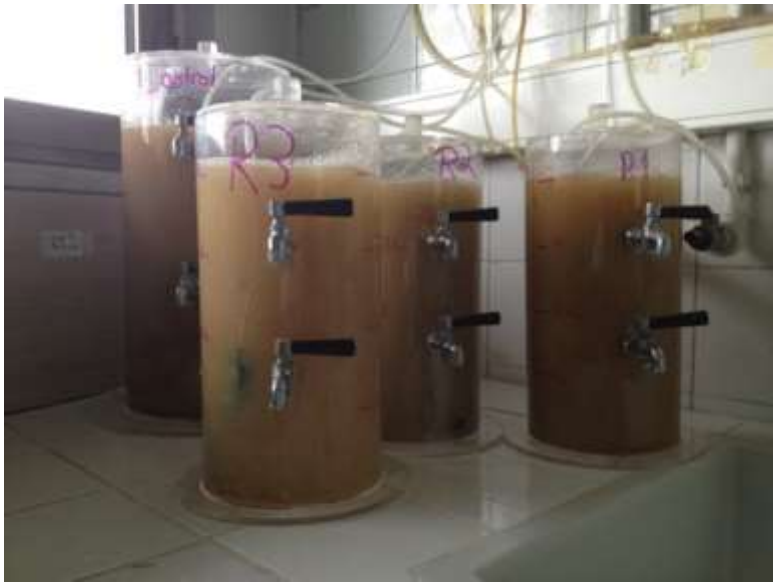


Figure 5.1. Activated sludge reactors in Part I: R1 (COD/TKN:10), R2 (COD/TKN:5), R3 (COD/TKN:0).

These reactors were fed every second day with a synthetic wastewater. To all feeds, alkalinity was added for complete nitrification. Peptone water, glucose and acetate were used as organic substances in feeds. Peptone water contributed both to COD and TKN, while glucose and acetate contributed only to COD. Also ammonium sulphate was used as

a nitrogen source. The contribution of organic substances to COD and TKN was measured with analytical methods and checked with theoretical calculations. Buffer capacity was provided by phosphate addition and pH was kept around 7.5-8.0.

Before each feeding the supernatant of the last run was withdrawn and analyzed. In all runs, COD, NH_4-N, and pH were regularly measured. Mixed Liquor Suspended Solids (MLSS) and Mixed Liquor Volatile Suspended Solids (MLVSS) concentrations were measured weekly.

Reactor 1 (R1)- COD/TKN ratio: 10

Synthetic domestic wastewater 1 (Feed 1 in Table 5.1) was prepared as a stock solution having a COD/TKN ratio of 10. The COD/TKN ratio in this feed was selected in accordance with a typical domestic wastewater. The composition of this feed is shown in Table 5.1.

Feed 1 had a COD and TKN of 10000 mg/L and 1000 mg/L, respectively. Approximately 20 % of COD and 15 % of TKN came from peptone water. Ammonium sulfate in the feed contributed to most of the nitrogen (850 mg N/L) in the stock solution. Reactor 1 was fed with this solution by diluting 20 folds. The diluted solution had the strength of a typical domestic wastewater.

Reactor 2 (R2)- COD/TKN ratio: 5

Feed 2 had a COD of 5000 mg/L and TKN of 1000 mg/L. The composition of this wastewater is shown in Table 5.1. Approximately 7.5 % of TKN came from peptone water. Ammonium sulfate in the feed contributed to most of the nitrogen (975 mg N/L) in the stock solution. Reactor 2 was fed with this solution by diluting 20 folds. The diluted solution had the strength of a domestic wastewater which had a lower organic content, but the same TKN strength as Feed 1.

Reactor 3 (R3)- COD/TKN ratio: 0

The feed of R3 (Feed 3) consisted of inorganic compounds only and did not contain any organic carbon. Therefore, the COD/TKN ratio was zero. The feed was rich in terms of ammonium for enhancement of nitrifiers. This stock solution contained 1000 mg NH_4-N/L.

5.1.2. Part II: Reactors Receiving Different Substrate Types

In this part, the effect of substrate type on EPS production was studied. COD/TKN ratio was kept constant at 10. For this purpose, three different reactors were operated in parallel, namely CR, RG and RP, each representing a different activated sludge system. The configuration of these reactors is shown in Figure 5.2.



Figure 5.2. Activated sludge reactors in Part II (COD/TKN:10).

Control Reactor (CR)- COD/TKN ratio:10

From the beginning of the thesis, CR was operated as a backup reactor. It was fed with Feed 1 as shown in Table 5.1. Thus, this reactor received the same feed as R1.

Peptone Reactor (RP)- COD/TKN ratio:10

The Peptone Reactor (RP) was started up with the sludge taking from CR. Feed P was composed of minerals and peptone as the only organic substance. Feed P had a COD of 10000 mg/L and 100 % of it came from peptone water. Feed P had a TKN of 1000 mg/L

and 98 % of it came from peptone water. Reactor RP was fed with this solution by diluting 20 folds, the diluted solution had then the strength of a typical domestic wastewater and was rich in protein.

Glucose Reactor (RG)- COD/TKN ratio:10

The Glucose Reactor (RG) was also started up with the sludge taking from CR. Feed G was composed of minerals and glucose was the only organic substance. Stock Feed G had a COD of 10000 mg/L and TKN of 1000 mg/L. 100 % of COD came from glucose. Ammonium sulfate in the feed contributed to nitrogen (1000 mg N/L). Reactor RG was fed with this solution by diluting 20 folds; the diluted solution had the strength of a typical domestic wastewater and was rich in carbohydrate.

5.1.3. Part III:Addition of AgNP to Reactors (Part II) Receiving Different Substrates

In this part, two new reactors were started up by taking sludges from RP and CR reactors. These new reactors were named as RP_{AgNP} and $CR_{AgNP.}$ The main aim here was to evaluate the effect of AgNP on the performance of activated sludge reactors when fed with different organic carbon sources. These reactors were operated over 250 days. AgNP was added to the reactors with the feed in each feeding period. The operation conditions were the same as in the main reactors (CR and RP). The configuration of these reactors is shown in Figure 5.3.



Figure 5.3. Configuration of reactors in Part III (COD/TKN:10).

5.2. Optimization of EPS Extraction

After an extensive literature review, possible extraction and measurement methods were tested. Optimization of methods took approximately 10 months. The selected EPS extraction and measurement techniques are presented below. Figure 5.4 presents schematically the steps in EPS extraction and analysis.

- *Concentration:* A sludge having a MLVSS concentration of 500 mg/L was taken from the reactor and centrifuged at 12,000xg and 4°C for 10 min, by using Beckman Coulter, Allegra 64R High-speed Refrigerated Centrifuge. The supernatant was filtered through 0.45 μm cellulose acetate syringe filter and stored at -18°C for further analysis.

- *Washing step:* After the concentration step, centrifuge tubes were filled with deionized water and centrifuged at 12,000xg and 4°C for 10 min. The supernatant was discarded in order to remove the ions coming from the reactor.

- *Extraction of the Very Loosely Bound-EPS (VLB-EPS)*: After the washing step, pellets were collected in a tube; then 30 mL of phosphate buffer was added (PBS, Table 5.2) and vortexed. After mixing, sludge was homogenized at 16,000xg, 2min. by using Heidolp Silent Crusher Homogenizer. By distrupting the flocs, VLB-EPS was released into the surrounding. The suspension was diluted to 50 mL and centrifuged at 29,000xg and 4°C for 20 min. The supernatant was filtered through 0.45 μm cellulose acetate syringe filter and stored at -18°C for further analysis. As explained in the next section (5.2.1), identification of the VLB-EPS fraction is a novelty of this study.

Table 5.2. Composition of PBS used in EPS extractions

Name	Concentration
$Na_3PO_4.12H_2O$	2 mM
$NaH_2PO_4.\ 2H_2O$	4 mM
NaCl	9 mM
KCl	1 mM

- *Extraction of the Loosely Bound-EPS (LB-EPS)*: Then, pellets were collected again in a tube, 30 mL of PBS was added and vortexed. After mixing, sludge was homogenized at 16,000xg for 2min. Suspension was diluted to 50 mL and poured in an Erlenmayer flask. The suspension was inserted to the Bandelin Sonorex ultrasonic water bath at 50W for 3 min. Then, the suspension was stirred at approximately 500 rpm for 10 minutes. Suspension was diluted to 50 mL and centrifuged at 29,000xg and 4°C for 20 min. Supernatant was filtered through 0.45 μm and stored at -18°C for further analysis.

- *Extraction of the Tightly Bound-EPS (TB-EPS):* TB-EPS extraction was performed by using Dowex® Marathon™ C sodium form (Sigma-Aldrich). Since Dowex was not totally clean, it should be washed with PBS for 1 hour before starting the extraction. 35 g washed, drained and filtered DOWEX was added to an Erlenmayer flask. Pellets from previous step were combined in a tube, 30 mL of PBS was added and vortexed. After mixing, sludge was homogenized at 16,000xg for 2 min. The suspension was diluted to 100 mL and poured into an Erlenmayer flask containing DOWEX. The Erlenmayer flask was wrapped with aluminium folio to keep the sample under dark and stirred at 800 rpm and 4°C for 4 hours. Then, sample was poured into centrifuge tubes. In order to remove the DOWEX from the suspension, there was an short spin performed at 29,000xg and 4°C for 2 min. Supernatant was poured to the new tubes and centrifuged at 29,000xg and 4°C for 20 min. Supernatant was filtered through 0.45 μm and stored at -18°C for further analysis.

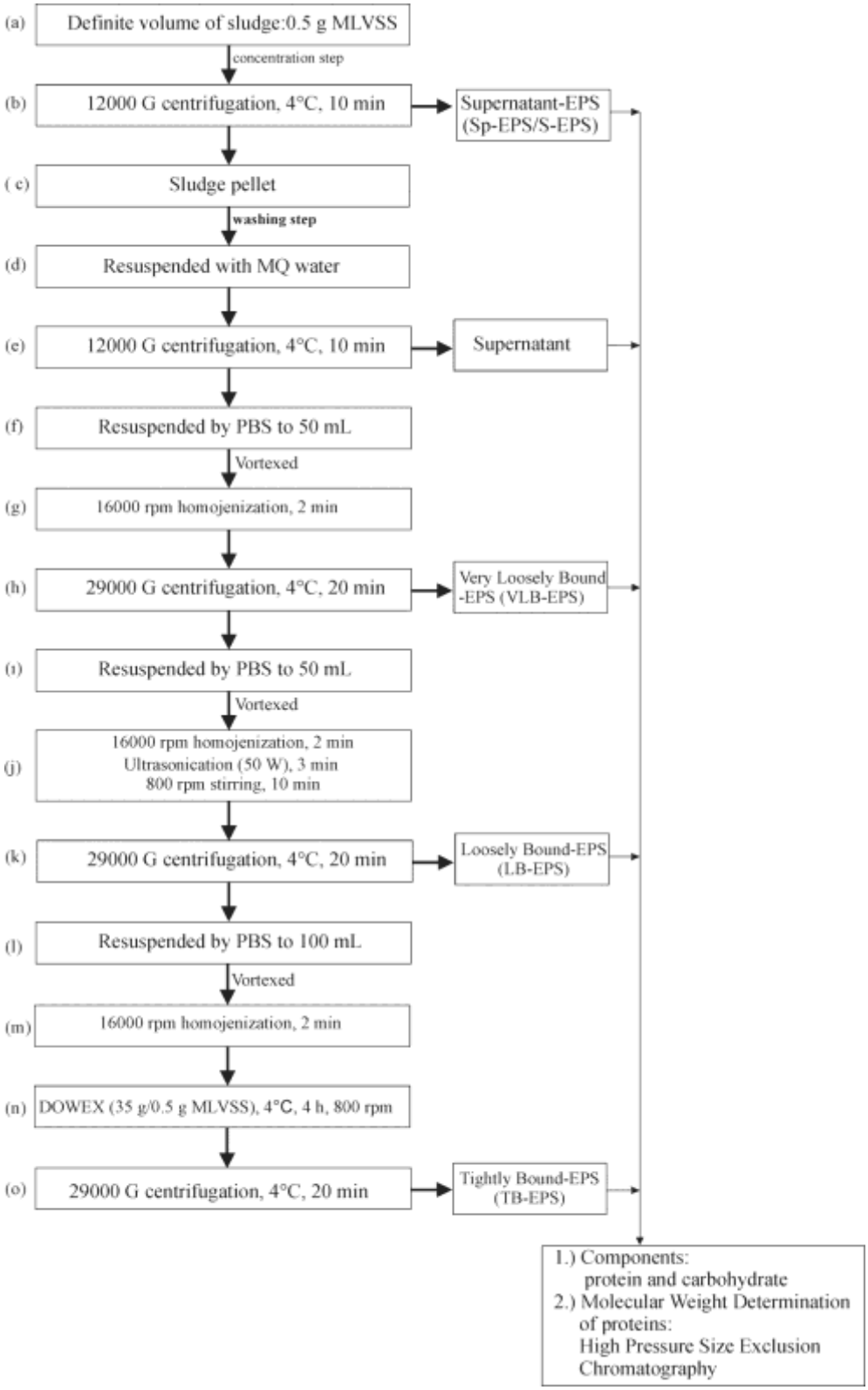


Figure 5.4. Schematic diagram of EPS extraction and analysis.

- *Blank:* In colorimetric measurements, PBS was used as a blank for the analysis of VLB-EPS and LB-EPS fractions. 35 g washed, drained and filtered DOWEX was

added to an Erlenmayer flask to which 100 mL of PBS was added and stirred at 800 rpm, 4°C for 4 hours under dark. Then, the sample was poured into centrifuge tubes and suspension was centrifuged at 29,000xg and 4°C for 20 min. The supernatant was filtered through 0.45 μm and stored at -18°C to be used as a blank for TB-EPS.

5.2.1. A Different Approach to EPS Fractionation

As a novel procedure, during the course of this study, as shown in Figure 5.5, a new EPS fraction was successfully differentiated, named as the **Very Loosely Bound-EPS (VLB-EPS)** (Geyik and Çeçen, 2015). In literature, this fraction is often disregarded or considered as soluble-EPS. In fact, VLB-EPS should not be regarded as soluble since they require some mechanical steps to detach from sludge structure. Alternatively, in other studies this fraction is considered within the scope of LB-EPS (Li and Yang, 2007; Wang et al., 2013). In such a case, however, this might lead to an overestimation of LB-EPS. As shown in Figure 5.5, the VLB-EPS fraction lies in between S-EPS and LB-EPS. Also this fraction might be of importance in holding flocs together as the more tightly bound fractions.

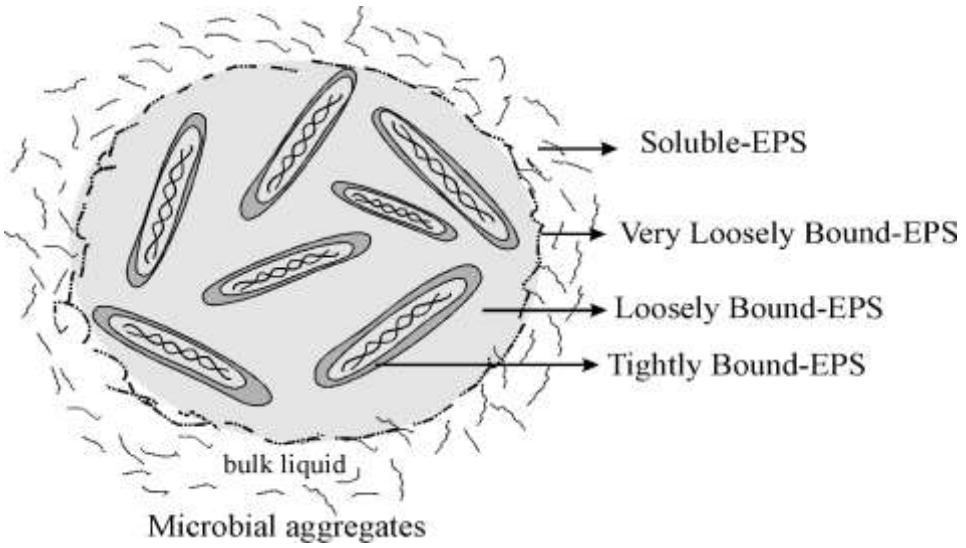


Figure 5.5. Fractionation of Extracellular Polymeric Substances (Geyik and Çeçen, 2015).

5.3. EPS Measurement Techniques

5.3.1. Analytical Methods

5.3.1.1. Phenol-Sulfuric Acid Method for Determination of Total Carbohydrates. The phenol–sulfuric acid method is a simple and rapid colorimetric method to determine total carbohydrates in a sample. The method detects virtually all classes of carbohydrates, including mono-, di-, oligo-, and polysaccharides. However, the absorptivity of different carbohydrates varies. Determination of sugars using this method is based on the measurement of absorbance of a colored aromatic complex at 490 nm formed between phenol and carbohydrate. The amount of sugar present is determined by comparison with a calibration curve using a spectrophotometer. Under proper conditions, the phenol-sulfuric acid method is accurate to ±2%. (DuBois et al., 1956).

Procedure. In order to prepare 5% of phenol solution, 50 g phenol (reagent grade) was dissolved in 1 liter of deionized water. EPS samples were taken from the freezer and thawed. Samples were vortexed and 1 mL of sample was inserted into a 16x100 mm glass tube. 1 mL of 5% of phenol solution and 5 mL of concentrated H_2SO_4 were added to the tube and vortexed. In order to speed up the reaction, samples were inserted into VELP Thermoreactor and digested at 100°C for 30 minutes. When samples were cooled down, the absorbance was read at 490 nm. Glucose solution was used as a standard in the range of 0-100 mg/L.

5.3.1.2. Lowry Method for Determination of Total Proteins. The method combines the reactions of copper ions with the peptide bonds under alkaline conditions (the Biuret test) with the oxidation of aromatic protein residues. The Lowry method (Lowry et al., 1951) is best used with protein concentrations of 0.01–1.0 mg/mL. It is based on the reaction of Cu^{+2}, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent Ciocalteay phosphomolybdicphosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. The Lowry method is sensitive to pH changes. Therefore, the pH of assay solution should be maintained at 10-10.5. The concentration of the reduced Folin reagent is measured at 750 nm.

Procedure. EPS samples were taken from the freezer and thawed. Then, they were vortexed. 0.5 mL of sample was inserted into 16x100 mm glass tube. 0.7 mL of Lowry solution was added.

Solution A (alkali reagent, for 500 mL)

2.8598 g NaOH

14.3084 g Na_2CO_3

Solution B (for 100 mL)

1.4232 g $CuSO_4.5(H_2O)$

Solution C (for 100 mL)

2.85299 g $Na_2C_4H_4O_6.2H_2O$

Lowry Solution (Daily)

In order to prepare the Lowry Solution, Solutions A, B and C were mixed in ratio of 100:1:1 (v:v). Then, 0.7 mL was added to the tubes. Samples were vortexed and incubated for 20 minutes at room temperature under dark conditions.

Folin-Ciocalteau reagent

5 mL of 2N Folin-Ciocalteau reagent were pipetted and added to the flask containing 5 mL of deionized water from which 0.5 mL were added to the tubes. Samples were gently vortexed and incubated for 30 minutes at room temperature under dark conditions. BSA was used as a standard. A calibration curve was prepared in the range of 0-100 mg/L. The concentration of the reduced Folin reagent was measured at 750 nm.

5.3.2. High Pressure Size Exclusion Chromatography (HPSEC)

The chromatographic fingerprints of extracted EPS samples were obtained by using Size-Exclusion Chromatography (SEC) with the Agilent Zorbax Bio Series GF-250 column. The properties of the column used in analysis are presented in Table 5.3.

Table 5.3. Conditions in the protein analysis using HPSEC.

Analytical Conditions	
Injection volume	50 µL
Mobile phase	20 mM Sodium phosphate buffer + 130 mM NaCl
Flow	1 mL/min
Dedector	Diode Array
Wavelength (nm)	257-264-210-230-280
Operation time	15 min
Column	Agilent Zorbax Bio Series GF-250, 250 mm x 9.4 mm

The calibration curve was generated by using Gel Filtration Markers Kit for Protein Molecular Weights 29–669 kDa (Sigma-Aldrich). Molecular mass and concentration of protein standards are presented in Table 5.4.

Table 5.4. Properties of protein standards used in HPSEC measurements.

Components	**App. Molecular Mass (kDa)**	**Injected concentration (mg/mL)**
Carbonic Anhydrase from bovine erythrocytes	29	3
Albumin, bovine serum	66	10
Alcohol Dehydrogenase from yeast	150	5
β-Amylase from sweet potato	200	4
Apoferritin from horse spleen	443	10
Thyroglobulin, bovine	669	8

5.4. Surface Charge and Hydrophobicity Measurements

Using the same sludges as in EPS extractions, also hydrophobicities and surface charges were determined. To measure surface charge and hydrophobicity, a detailed literature survey was first done (Benoit et al., 1998; Shin et al., 2000; Liao et al., 2001;

Durmaz and Sanin, 2003; Garikipati, 2005; Saini, 2010)**.** Then, the following methods were selected. Detailed information can be found in a former MSc. Thesis (Kılıç, 2014) and the paper in press (Geyik et al., in press).

5.4.1. Surface Charge

In determination of surface charge, the colloidal titration method was used with minor modificiations (Liao et al., 2001; Durmaz and Sanin, 2003; Garikipati, 2005). For this purpose, 10 mL of washed sludge was diluted to 100 mL with deionized water. The pH was adjusted to 7.5 mL. 0.001 N of polybrene solution and a few drops of Toluidine blue indicator were added. The solution was titrated with 0.001 N of potassium polyvinylsulfate (PVSK) solution until the color changed from blue to pink/purple. The PVSK solution was standardized against Zephiramine solution. All surface charge analysis were done in duplicate and the average values were reported. The surface charge of sludge was calculated by using the following formula:

$$\text{Surface Charge } (\text{meq}/\text{g SS}) = \frac{(\text{A-B}) \times \text{N} \times 1000}{\text{mL of samples x } \frac{\text{mg}}{\text{L}} \text{ SS}} \times 1000 \qquad (5.1)$$

where;
A: mL of PVSK used for sample,
B: mL of PVSK used for blank,
N: the normality of PVSK.

5.4.2. Hydrophobicity

Using the same sludge samples as in surface charge determination, hydrophobicity analyses were done. In hydrophobicity measurements, the octane adhesion test method was used with minor modifications (Benoit et al., 1998). For this purpose, the absorbance of the sample was initially adjusted to nearly 0.3 at 600 nm. Then, 10 mL of sample and 4 mL n-octane solution were mixed. The suspension was vortexed for 2 minutes and settled for 10 minutes for phase separation. From the aqueous supernatant, a sample was withdrawn and

the optical density (OD_{600}) was measured at 600 nm. This was reported as the final optical density. All analyses were done in duplicate and the average values were reported. The hydrophobicity of sludge was calculated by using the following formula:

$$\text{Hydrophobicity (\%)} = \left(1 - \frac{\text{Abs final}}{\text{Abs initial}}\right) \times 100 \quad (5.2)$$

5.5. Effect of Inhibitiory Metals on EPS Production

5.5.1. Short-Term Inhibition Experiments in Respirometry Flasks

In order to determine the inhibitory effect of Ag^{+} and AgNP on activated sludge, respirometry was used as a tool. The response of biomass to heavy metals, namely, the inhibitory effect on biomass, was measured using the Columbus ER-10 Respirometry (Figure 5.6). In inhibition tests, ten respirometric chambers were operated as bioreactors where two of them were chosen as control reactors. The respective feed and silver were added to chambers. Before addition of sludge, pH was adjusted to prevent exposure of biomass to extreme pH. Then, chambers were put into a constant temperature bath mechanical shaker at 25 ºC, 120 rpm, for 24h.

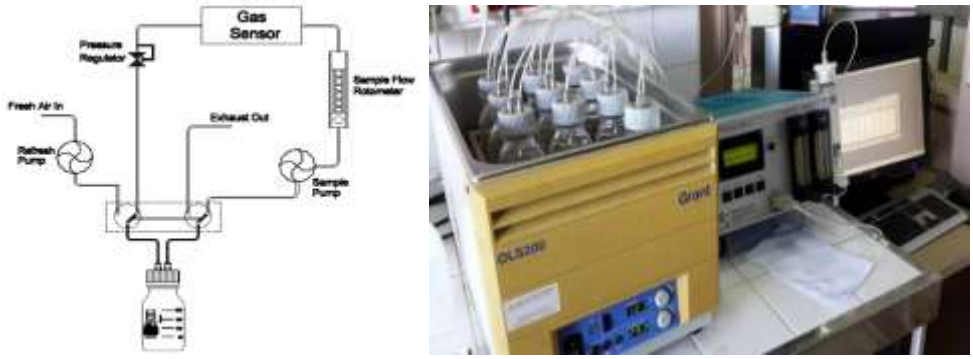


Figure 5.6. Schematic representation of respirometric measurements.

Each inhibition test was performed in approximately 21 hours. Under the initial and final conditions of tests, also pH, COD, NH_4-N, MLVSS, MLSS and EPS were monitored.

5.5.2. Long-Term Inhibition Experiments in Semi-Continuously Fed Batch Reactors

Sludge samples were taken from CR and RP reactors and new reactors were started up in order to examine the effect of long-term AgNP addition on EPS production. These new reactors were named as CR_{AgNP} and RP_{AgNP}. The reactors were operated in parallel to CR and RP. The whole reactor operation was separated into four phases. Details of these phases are presented in Sections 6.3.3.1 and 6.3.3.5.

During reactor operation, EPS, COD, MLSS and MLVSS were monitored. Moreover, every week respirometric tests were performed in order to observe the inhibitory behavior of AgNP.

5.6. Synthesis and Characterisation of Nanosilver (AgNP)

The nanosilver stock suspension was prepared according to the chemical reduction method with minor modifications (Zhang et al., 2011). In 32 mL of ultra pure water, an aqueous solution of silver nitrate (0.05 M, 1 mL), trisodium citrate (75 mM, 10 mL) and poly vinylpyrrolidone (PVP) (average molecular weight MW ~ 10, 000 g/mol, 17.5 mM, 2 mL) were combined and vigorously stirred at room temperature. Sodium borohydride ($NaBH_4$, 100 mM, 5 mL) was rapidly injected into this mixture for formation of nanoparticles. The suspension was mixed at 800 rpm for 3 minutes. The AgNP suspension prepared by this method was transparent and had a bright yellow color. The steps are illustrated in Figure 5.7.

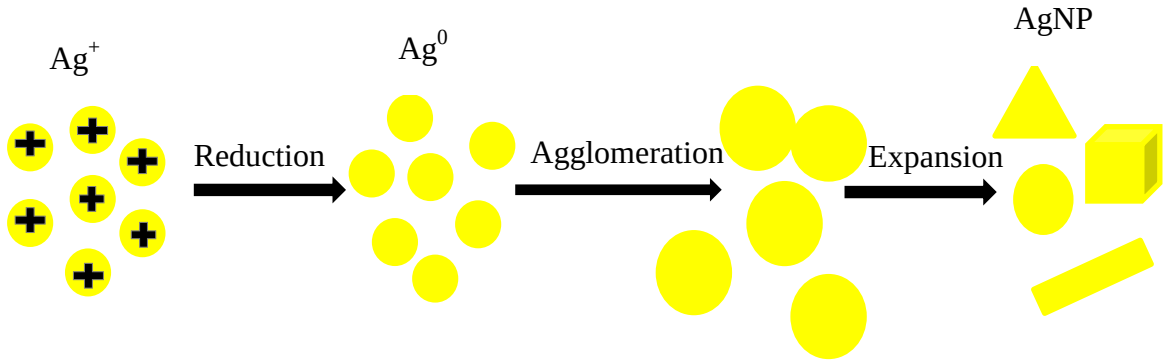


Figure 5.7. Steps in AgNP synthesis (Kittler, 2009).

In the presence of PVP, the transition from the light yellow to deep yellow color took about 30 min. The suspension was characterized by a HACH UV-visible spectrophotometer for unique surface plasmon absorption band in the presence of nanosilver. The maximum absorbance in the solution was observed at 400 nm, indicating successful synthesis of AgNPs (Choi et al., 2008). Further, the stability of synthesized AgNP was monitored over 2 months. The size distribution of AgNP was determined by Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) in Switzerland, at the Swiss Federal Institute of Aquatic Science and Technology (EAWAG). Besides, the hydrodynamic diameter and zeta potential (Z-potential) of fresh AgNP suspension and the one stored at room temperature for 2 months were determined using Brookhaven's NanoBrook 90Plus particle size analyzer in Boğaziçi University at the Institute of Environmental Sciences and the Advanced Technologies R&D Center, respectively.

5.6.1. Determination of Total AgNP Concentration in Reactors

To prepare samples for AAS analysis, 10 mL of the sample was digested with 4.5 ml of 30% HCl and 2.0 ml 65% HNO_3 using a hot plate acid digestion. HCl was chosen for digestion of AgNP to promote the formation of a soluble $[AgCl_x]^{1-x}$ complex with an excess of Cl^- ions as indicated by Kaegi et al. (2013). Due to the lack or lower Cl^- concentration in the wastewater, AgCl precipitation and loss of analyte would occur when acidified with HNO_3. Measurements were performed against an external calibration using a Fluka ICP standard in the low (0.03 mg/L-1 mg/L) and high range (1 mg/L–5 mg/L) concentrations.

5.7. Monitoring Complexation of EPS with Ag^+ and AgNP by Using Voltammetry

The voltammetric/polarographic method was used in order to measure the free Ag^+ and weak labile Ag concentration in stock AgNP solution as well as in EPS fractions. For this purpose, Metrohm VA 757 computrace having a solid state electrode (SSE) was used and Ag and AgNP were measured by using the Metrohm method (VA Application Work AW DE4-0204-122007, 2007). A conventional three-electrode arrangement: a rotating disc

electrode (RDE) with glassy carbon tip; an Ag:AgCl electrode with double junction was used as the reference; and a platinum counter electrode was used as an auxiliary electrode.

In order to measure Ag^+/AgNP, 2 mL of Ag^+/AgNP sample and 10 mL of supportive electrolite were present at pH 4.5 in the measuring cell. The solutions used in VA measurements are listed in Table 5.5. The measurement parameters of voltammetry are presented in Table 5.6.

Table 5.5. Solutions used in VA measurements.

Solutions	**Preparation**
Na_2EDTA solution	c = 0.2 mol/L 7.44g $Na_2EDTA.2H_2O$ are weighed in 100 mL volumetric flask and dissolved in ultrapure water
Supportive Electrolyte (KNO_3 + Na_2EDTA) c(KNO_3) = 0.2 mol/L c(Na_2EDTA)= 4mmol/L	2.02 g KNO_3 and 2 mL Na_2EDTA (c = 0.2 mol/L) are added in a 100 mL volumetric flask and filled up to the mark with ultrapure water
Ag^+ standard solution = 50 mg/L	5 mL Ag stock solution was added to a 100 ml volumetric flask , acidified with 100 µL 65% HNO_3 and filled up to the mark with ultrapure water

Binding of AgNP and Ag^+ to different EPS fractions was also studied. EPS was uncontacted with either AgNP or Ag^+ before. In the measuring cell, 2 mL of EPS sample, 10 mL of supportive electrolite at pH 4.5 and a definite volume of Ag^+ or AgNP solution were added to obtain 2 mg/L inside the cell. After the addition of metal, the solution was stirred at 250 rpm for 15 min to allow complexation of EPS with the metal. Then, the concentration of metal in the sample was determined by two standard additions using 50 mg/L standard Ag^+ solution. The peak potential of Ag^+ was located at 0.25 mV.

Table 5.6. The parameters in voltammetric measurements.

Specifications	**Parameters**
Electrode Type	RDE-GC
Drop size	No drop
Measurement Mode	DP Differential Pulse
Calibration technique	Standard addition
Stirrer Speed	2000 rpm
Addition	manual
Determination of Ag	
Sample amount	2.0 mL
Cell volume	12.0 mL
Measure blank	No
Addition purge time	10 s
No. of additions	2
No. of replications	2
Voltammetric parameters	
Initial purge time	300 s
Pretreatment	
Deposition Potential	-0.4V
Deposition time	60 s
Equilibration time	5 s
Sweep	
Start potential	0.0 V
End potential	0.45 V
Pulse amplitude	50 mV
Pulse time	40 ms
Voltage step	4 mV
Voltage step time	0.1 s
Sweep rate	40 mV/s
Cell off after measurement	Yes
Substances	
Peak Evaluation	Area
Scope	Whole Peak
Peak potential Ag approx.	0.25mV

5.8. Investigation by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

AgNP suspension was investigated using TEM and SEM as indicated Kaegi et al., 2013. TEM grids were prepared by placing a drop of suspension (mixed liquor or supernatant) on a holey carbon grid (Cu 200 mesh, holey carbon coated, Plano GmbH, Germany) and drawing the suspension through the TEM grid using a paper tissue. The

TEM grids were washed afterwards in a drop of distilled water to remove the dissolved components. The samples were then investigated either in a SEM (Nova NanoSEM 230, FEI) operated at either high or low vacuum conditions or in a TEM (Tecnai F30 STEM, FEI; HD-2700-Cs, Hitachi).

5.9. Statistical Analysis

Statistical analyses were conducted using GraphPad Prism, 6.0 for Windowns demo version. IC_{50} values of Ag^{+} and AgNP were calculated by using nonlinear fitting. To determine the statistical significance of differences between the samples, the one-way ANOVA test was applied. Then, the student's t-test was conducted. Significant differences were determined at $p < 0.05$.

6. RESULTS AND DISCUSSIONS

6.1. Part I:Effect of the COD/TKN Ratio on EPS Production

In the first step of this dissertation, the effect of the COD/TKN ratio on composition and fractionation of EPS was examined. In a full-scale wastewater treatment system, composition and strength of influent wastewater may fluctuate with respect to time. In particular, changes may occur in the COD/TKN ratio. Such changes might have a great influence on microbial ecology of sludge. Possibly, during adaptation of microorganisms to a new feeding condition, also variations are expected in fractionation and composition of EPS (Yang and Li, 2009; Miqueleto et al., 2010; Ye et al., 2011).

Often, most studies do not mention whether EPS results were achieved under steady- or transient conditions. Therefore, this part of the study about EPS production was divided into two as;

(i) stabilization period (Geyik and Çeçen, 2015)
(ii) steady-state period (Geyik and Çeçen, 2014a).

As indicated in Section 5.1.1, activated sludge was initially taken from the recycle line of the Paşaköy Advanced Biological Wastewater Treatment Plant. In the first phase, a single activated sludge reactor ("main reactor") was started up. When this reactor reached steady-state conditions with respect to MLSS and MLVSS, the sludge was divided into different reactors. Three reactors, namely R1, R2 and R3 reactors were then started up at different COD/TKN ratios, namely at 10, 5 and 0, respectively.

The whole operation of these reactors lasted around 750 days. The stabilization period regarding EPS stabilization covered the 0-250 days of operation whereas the steady-state period covered the period, from 250 to 750 days. Details about reactor operation can be found in former MSc.Theses (Ayyıldız, 2013; Kılıç, 2014).

6.1.1. EPS Production in the Stabilization Period

The main idea in the present study was to examine the dynamics of EPS production, namely the changes taking place in EPS until steady EPS production was reached. It was very likely that EPS production underwent gradually some changes as indicated in following sections. Moreover, stabilization of MLVSS and substrate removal (COD or ammonium) were also taken into consideration.

6.1.1.1. Monitoring Biomass Concentration in Reactors. As seen in Figure 6.1a and 6.2a, upon feeding with synthetic feeds, biomass (MLVSS) concentrations decreased sharply in reactors. The most dramatic decrease in MLVSS was observed in R3 sludge which did not receive any organic carbon (COD/TKN=0). Probably, the absence of organic carbon led soon to a loss of heterotrophic biomass in the initial periods of operation. In R1 and R2 reactors receiving organic matter, the decreases in MLVSS were not as severe as in R3 and soon high levels were reached. Although substrate loading per reactor volume remained constant in all reactors as shown in Figure 6.1b and 6.2b, stabilization of MLVSS took a long period of time. Finally, MLVSS in reactors were statistically different from each other (p<0.05) and followed the order R1>R2>R3 (R1: 3023±667 mg/L; R2: 2253±726 mg/L; R3: 1141±576 mg/L). Thus, the highest MLVSS was kept in the R1 reactor that was operated at the highest COD loading rate, as also reported in literature (Shariati et al., 2011).

6.1.1.2. Substrate Removal in the Stabilization Period. After start-up of each reactor with the respective feed, steady removal of COD and ammonium were soon achieved. At least 90 % of influent COD was removed in R1 and R2, whereas ammonium removal exceeded 90 % in the nitrification reactor, R3. Since fluctuations were observed in MLVSS in the initial periods of operation, loading and removal rate expressions seemed to vary. At about 150-200 days, in R1 and R2, MLVSS stabilized. Then, COD loading and removal rates were stabilized too (Figure 6.1b-c).

Also in R3, initially a sharp decrease was observed in MLVSS (Figure 6.2a). Therefore, NH_4^+-N loading and removal rate expressions per biomass seemed to be higher. After about 150-200 days, MLVSS concentration stabilized at around 1000 mg/L MLVSS.

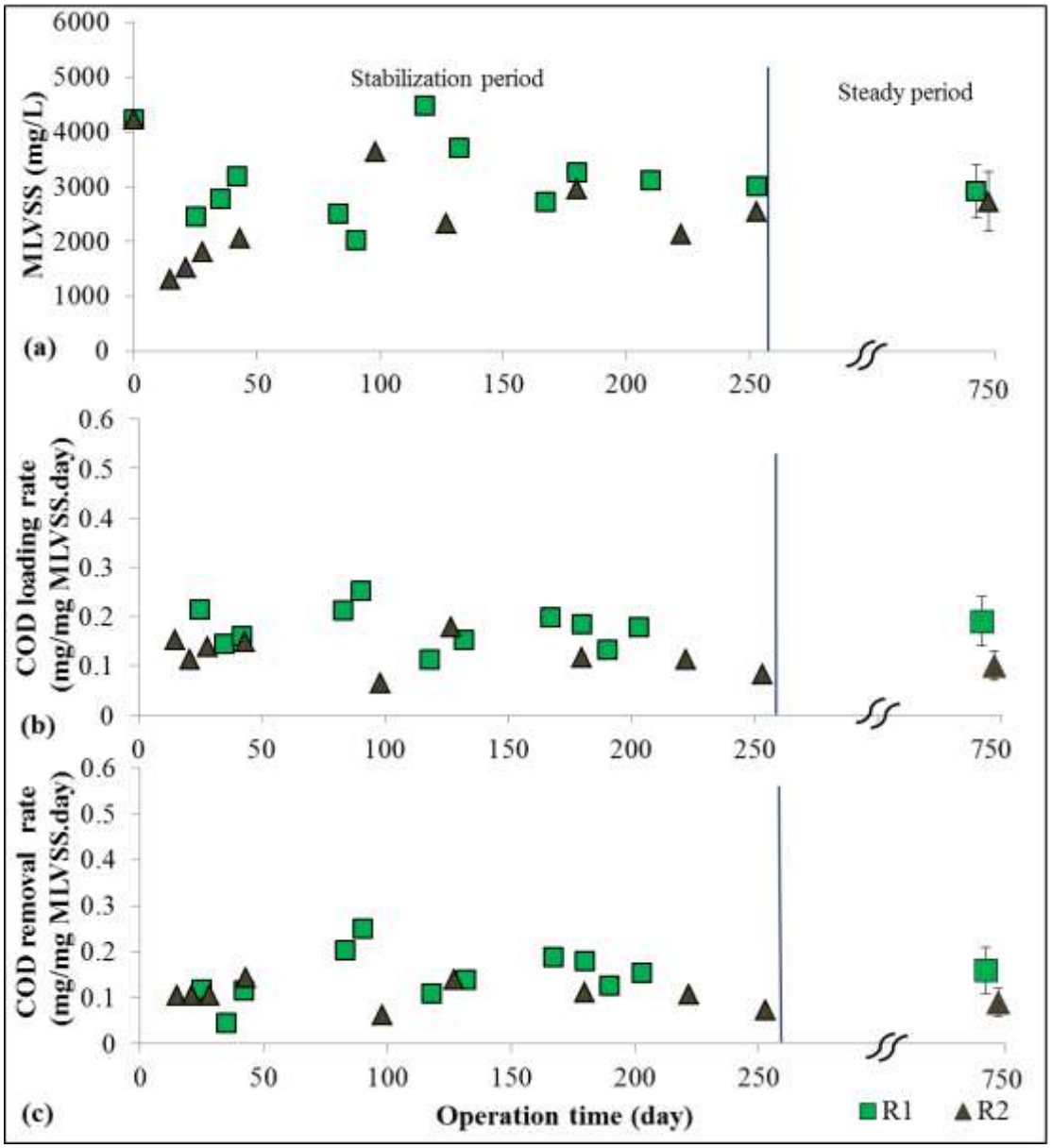


Figure 6.1. Reactor operation in the adaptation (stabilization period) and steady-state periods (stabilized period): a) Biomass (MLVSS) concentrations in R1 (COD/TKN 10) and R2 (COD/TKN:5) b) COD loading rates in R1 and R2, c) COD removal rates in R1 and R2 (Geyik and Çeçen, 2015).

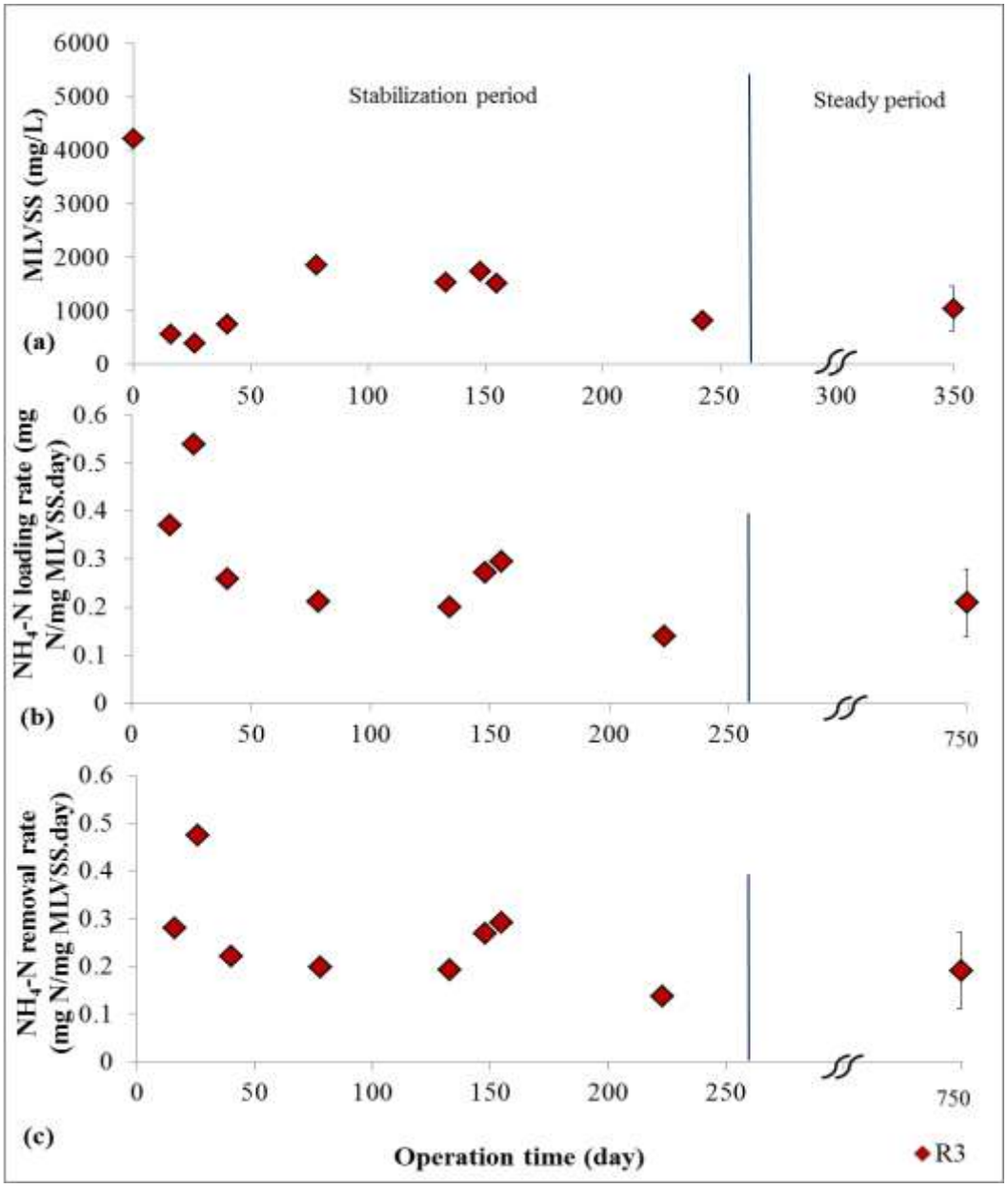


Figure 6.2. Reactor operation conditions in the adaptation period (stabilization period) and steady-state period (stabilized period) in the nitrifying reactor, R3: (a) Biomass (MLVSS) concentrations (COD/TKN:0) (b) NH_4-N loading rates, (c) NH_4-N removal rates (Geyik and Çeçen, 2015).

6.1.1.3. Bound-EPS Fractions in the Stabilization Period.

Total EPS yield. In general, the amount of EPS produced in each sludge correlated somehow with the MLVSS in the reactor. In addition, upon stabilization of MLVSS, also EPS production stabilized in reactors. The EPS yield is defined as the total-EPS produced per biomass. It is reported to vary with the type of wastewater, origin and microbial ecology of sludge, and operation parameters. Certainly, also EPS extraction is of importance. Yet, to date there is no standard method for EPS extraction. In our case, the maximum EPS yield was about 53 mg total-EPS/g MLVSS, which was lower compared with some literature studies (Liu and Fang, 2002b; Wilén et al., 2003b; Adav and Lee, 2008; Pellicer-Nàcher et al., 2013), reporting a range 57-544 mg EPS/g MLVSS. In the present study, harsh methods such as thermal or base treatment or chemical use (Liu and Fang, 2002b) were intentionally avoided because they might lead to overestimation of EPS due to interference of intracellular proteins and carbohydrates. Also, the dose of CER might affect the extraction of TB-EPS. In literature, this dose is reported to vary in a wide range (10-100 g DOWEX/gMLVSS) (Frølund et al., 1996; Dignac et al., 1998; Wuertz et al., 2001; Sesay et al., 2006; Kong et al., 2015). Since TB-EPS constitutes the backbone of EPS, variations in CER dose might bring about a great difference in EPS yield.

Major and Minor Fractions in EPS. Figure 6.3 provides a general look into the different fractions in EPS in the stabilization period. Figure 6.4 illustrates the range of major and minor fractions in EPS in the stabilization period (left figure) as well as the changes taking place with respect to time (right figure).

As seen in Figures 6.3 and 6.4, in all sludges TB-EPS production was much higher than VLB-EPS and LB-EPS. Approximately 75 % of total-EPS consisted of TB-EPS which constituted the *major fraction*. Therefore, in each sludge variations seen in total-EPS depended largely on this fraction (Figure 6.4). As seen in Figure 6.4, in R3 sludge, TB-EPS and paralleling this total-EPS, were very stable from the beginning, indicating a more rapid stabilization upon contact with Feed 3. On the other hand, in other sludges, obvious fluctuations and a decreasing trend were noted, particularly in TB-EPS and total-EPS. After about 150 days of operation, stabilization was observed in all fractions (Figure 6.3 and 6.4).

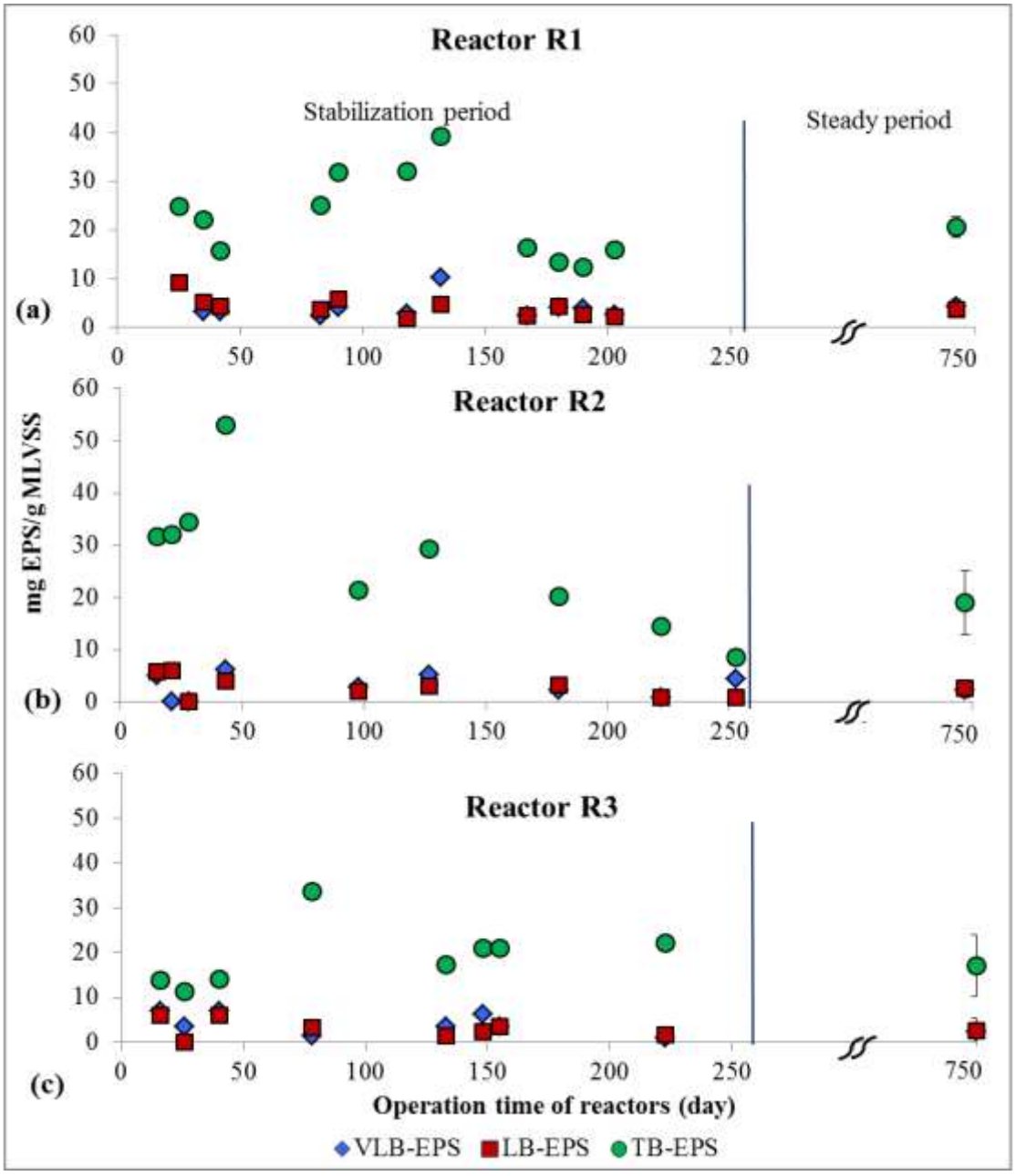


Figure 6.3. EPS fractions in R1, R2 and R3 sludges (VLB-EPS: Very Loosely Bound-EPS; LB-EPS: Loosely Bound-EPS; TB-EPS: Tightly Bound-EPS) (Geyik and Çeçen, 2015).

The production of the *minor fractions*, namely, VLB-EPS and LB-EPS was usually below 11 mg/g MLVSS. Statistically, the production of these fractions was at the same level in R1, R2 and R3 sludges (p>0.05).

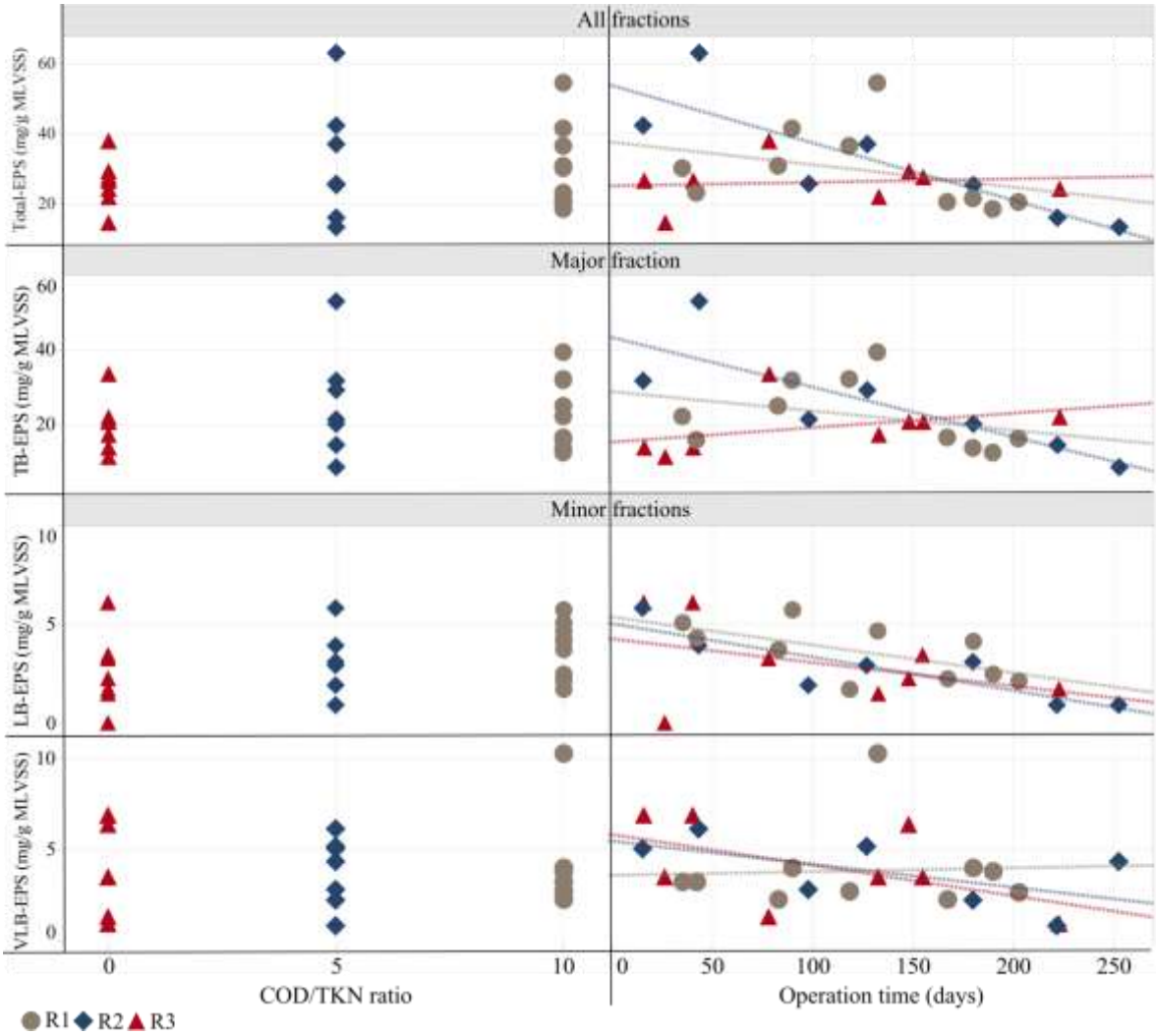


Figure 6.4. Detailed examination of the different fractions in EPS in activated sludges operated at the COD/TKN ratio of zero (R3 sludge), 5 (R2 sludge) and 10 (R1 sludge). Left: The spread in total-EPS in the stabilization period, Right: The variations in EPS withrespect to stabilization period. (VLB-EPS:Very Loosely Bound-EPS; LB-EPS:Loosely Bound-EPS, TB-EPS: Tightly Bound-EPS).

Regarding the VLB-EPS, slight decreases were noted with respect to time in R2 and R3 sludges whereas almost no change was observed in R1 that received a balanced feed composed of different organics. The VLB-EPS, a new fraction identified in this study, deserves special attention. As reported in Section 6.3.3, if biomass was exposed to silver species, excessive VLB-EPS production seemed to protect microorganisms from toxic effects. Besides, the VLB-EPS fraction had a greater complexation capacity when it was contacted with silver species (Geyik and Çeçen, 2014b).

When three sludges were compared with each other, the production of LB-EPS was statistically similar (p>0.05). In each sludge a gradual decrease was noted in LB-EPS with respect to time as seen in Figure 6.4. High production of LB-EPS is usually believed to lead to a slimy structure (Geyik and Çeçen, 2014c). Some literature studies also showed that LB-EPS had a negative effect on bioflocculation and sludge-water separation (Li and Yang, 2007; Wang et al., 2013). Until 25 days of operation, in some EPS extracts from R2 sludge, VLB-EPS and LB-EPS were found to be below the detection limit. The reason may be attributed to the utilization of these looser fractions at the lower organic loading rate in R2.

6.1.1.4. Changes in the Carbohydrate and Protein Content of EPS. As seen in Figure 6.5a-b, in R1 and R2 reactors fed with organics, protein-EPS production was similar until 30 days of operation. However, continuation of feeding led to some differentiation. Evidently, at later times of operation, in R2 sludge the relative decrease in protein-EPS became higher than in R1. In general, in both sludges carbohydrate-EPS production did not change much with respect to time. All data indicated that changes occurred in protein-EPS rather than carbohydrate-EPS. Generally, EPS composition stabilized at 150-200 days of operation when also stabilization was reached in MLVSS.

Although the origin of the sludge in the nitrifying reactor R3 was the same as R1 and R2, feeding with inorganic substances led very soon to a decrease in protein-EPS (Figure 6.5c), as noticed in early periods of operation. In this reactor, initially a high decrease was also noted in MLVSS. In particular, carbohydrate-EPS was reduced to noticeably lower levels compared with other sludges. Since R3 sludge received no organic carbon, bacteria might have utilized some of the EPS as a carbon source. Interestingly, however, the amounts of both protein- and carbohydrate-EPS were very stable from the beginning and the least EPS fluctuations were observed in this sludge (Figure 6.5). Obviously, the microbial ecology of this sludge became sooner stabilized than R1 and R2 that were fed with organic carbon. Also another study pointed to stable EPS production in a nitrifying sludge within a short period of time (Zhang et al., 2011).

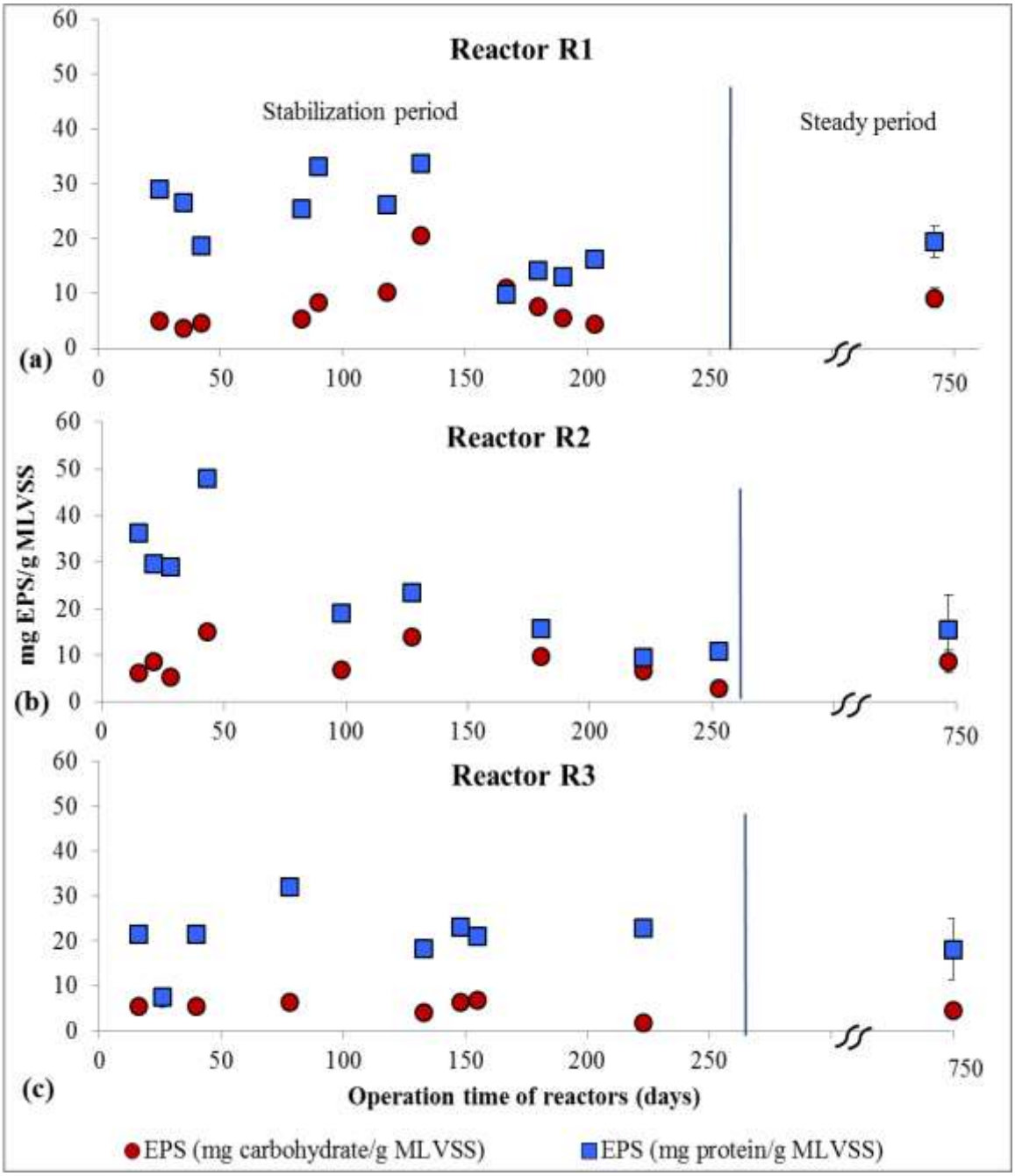


Figure 6.5. Production of protein- and carbohydrate-EPS in reactors (a) R1 (COD/TKN:10), (b) R2 (COD/TKN:5) and (c) R3 (COD/TKN:0) (Geyik and Çeçen, 2015).

The protein and carbohydrate content of each EPS fraction was significantly different from each other in R1 and R3 sludges (p<0.05). Although the COD/TKN ratio differed between R1, R2 and R3 sludges, in each case protein-EPS was dominant. Depending on the respective amount of proteins (P) and carbohydrates (C), the P/C ratio in EPS changed

too. The P/C is an important parameter affecting surface properties of sludge such as hydrophobicity and surface charge. Although in both R1 and R2, carbohydrates and proteins fluctuated in the stabilization period (Figure 6.5a-b), average values were very close to each other. Obviously, the differences in organic loading rates between R1 to R2 did not bring about a great change in EPS composition. However, in R3 sludge the P/C ratio was clearly higher than in others.

6.1.1.5. Changes in the Molecular Weight of Protein-EPS.

Chromatographic Properties. In interpretation of HPSEC results, fingerprints recorded at 210 nm were only taken into consideration since peaks at 260 and 280 nm were very weak. Also in protein standards peaks were mainly observed at 210 nm. According to literature, many amino acids like tryptophan, phenylalanine, tyrosine absorb light in the range of 180-230 nm (Scopes, 1974; Schmid, 2001).

One of the main problems in EPS extractions is the interference of intracellular material. DNA and RNA are reported to show a strong absorbance near 260 nm (Schmid, 2001). In the present work, hardly any absorbances were detected at 260 nm. This indicated that the EPS extraction methods used, namely homogenization, sonication and CER did not lead to cell disruption.

In reactors R1, R2 and R3, high MW proteins of 1209≤EPS≤1977 kDa were produced until about 150, 98 and 40 days of operation, respectively (Figure 6.6). Then, production of such proteins was no longer observed and EPS became relatively more stable. In literature such high MW protein-EPS are regarded as biomass-associated products (BAP) (Laspidou and Rittmann, 2002b). In the initial phases of EPS stabilization, there was also a drop in MLVSS levels. Therefore, most probably these high MW proteins emerged due to lysis of some microorganisms.

In all EPS fractions, a great portion of protein-EPS had a MW≤12.5 kDa. These smaller proteins became even more dominant near the end of the stabilization period. The main finding of this study was that in each sludge proteins differed. For example, proteins having MWs of 12.0≤EPS≤12.5 kDa were produced only in R1 sludge (Figure 6.6). This is

thought to arise due to the high microbial variety in R1 sludge. This sludge was fed relatively with a high amount of organics supporting the growth of a higher number of microorganisms than in other reactors.

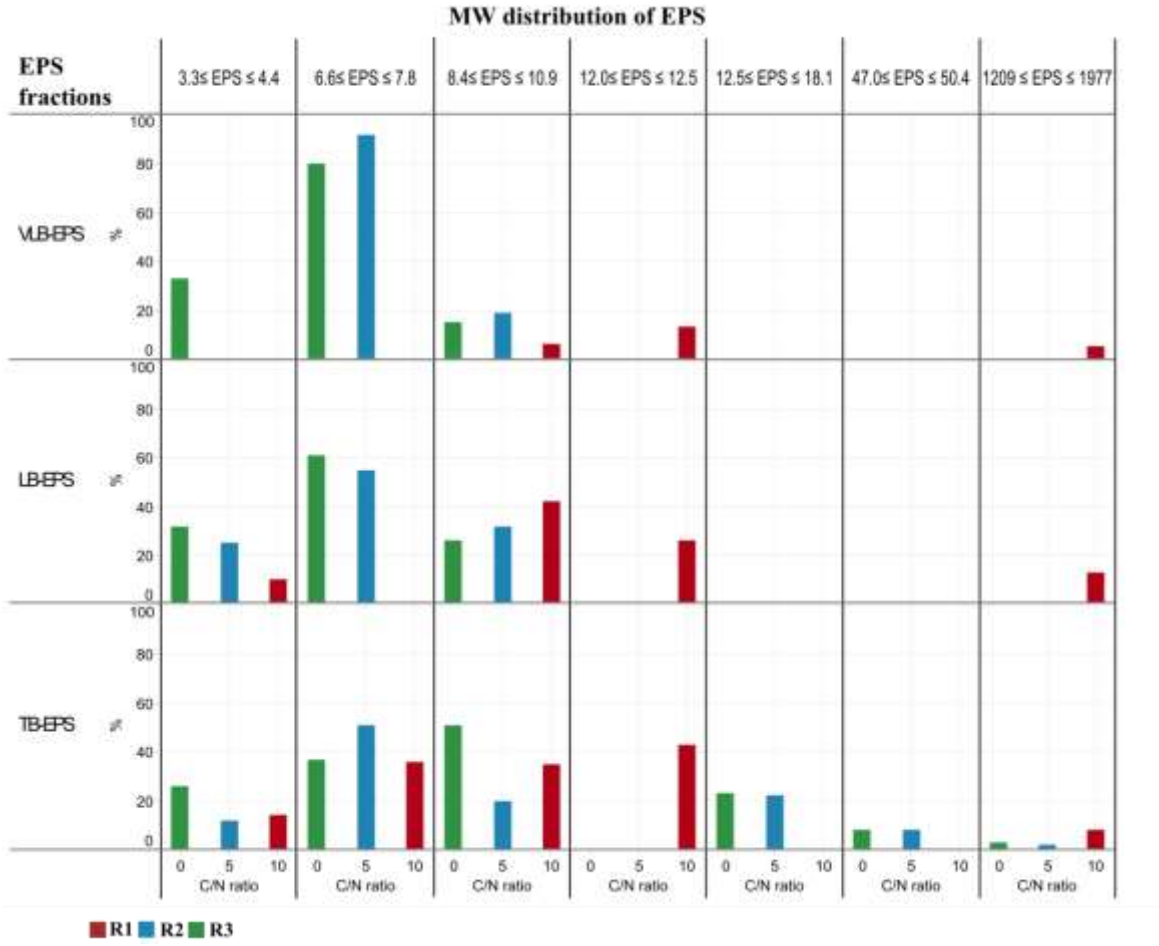


Figure 6.6. MW distribution in protein-EPS in the stabilization period (Geyik and Çeçen, 2015).

The second finding was that EPS stabilized earlier in the nitrifying sludge R3 that was operated at the COD/TKN of zero. EPS stabilization is possibly also related with the type of substrates. In this sludge fed with ammonium alone, nitrifiers became enriched and activities of other microorganisms were decreased. Therefore, this might be a factor for rapid stabilization of EPS.

6.1.2. Achievement of Steady-State in EPS Production

6.1.2.1. Monitoring Performances of Reactor R1, R2 and R3 in the Steady Period. In long-term operation, nearly steady-state conditions were achieved in all R1, R2 and R3 reactors in terms of effluent COD, nitrogen and biomass concentrations. In R1 reactor the average organic loading rate was about 0.19 mg COD/mg MLVSS.day. The initial COD at the start of semi-continuous runs was about 1000 mg/L whereas 90 % removal was observed. The average specific substrate removal rate was accordingly about 0.17 mg COD/mg MLVSS.day. In the R2 reactor, the average organic loading rate (0.11 mg COD/mg MLVSS.day) was lower than in R1 (Figure 6.7).

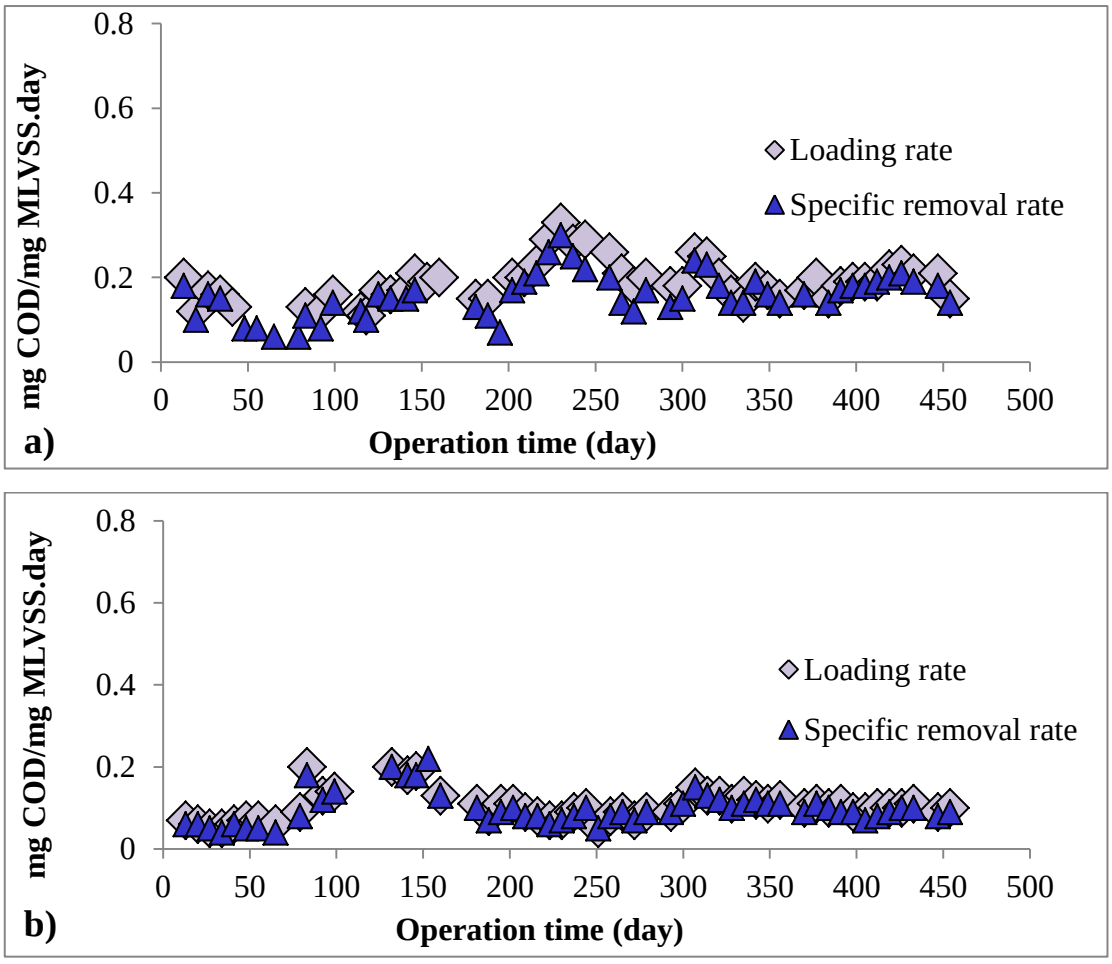


Figure 6.7. Organic loading and specific substrate removal rates a) Reactor R1 b) Reactor R2 (Geyik and Çeçen, 2014a).

The initial COD at the start of semi-continuous runs was about 500 mg/L whereas 92 % removal was observed. In both R1 and R2 reactors, nitrogen in the feed was used in cell synthesis as well as in nitrification. However, as also concluded from sludge respiration measurements (Çeçen and Kılıç, 2015), nitrification was more favored in the latter, since the COD/TKN ratio was lower than in R1.

In the nitrification reactor R3 (COD/TKN=0), in the period of EPS extractions, the ammonium loading rate was about 0.27 mg NH_4-N/g MLVSS.day (Figure 6.8). A great fraction of ammonium was removed and the average specific ammonium removal rate was 0.25 mg NH_4-N/mgMLVSS.d. In general, MLVSS concentration and MLVSS/MLSS ratio in a sludge depended on the COD/TKN ratio in the feed. In R3, a relatively low MLVSS concentration was maintained under steady-state conditions (at about 1000 mg/L) and the MLVSS/MLSS ratio dropped to about 0.30. Since this reactor was not supplied with organics, some heterotrophs were probably lost in long term. On the other hand, in R1 (COD/TKN:10) and R2 (COD/TKN:5) the MLVSS/MLSS ratios were about 0.80 and 0.68, respectively. Also, MLVSS concentrations were at much higher levels in R1 and R2, at about 2800 and 2200 mg/L, respectively.

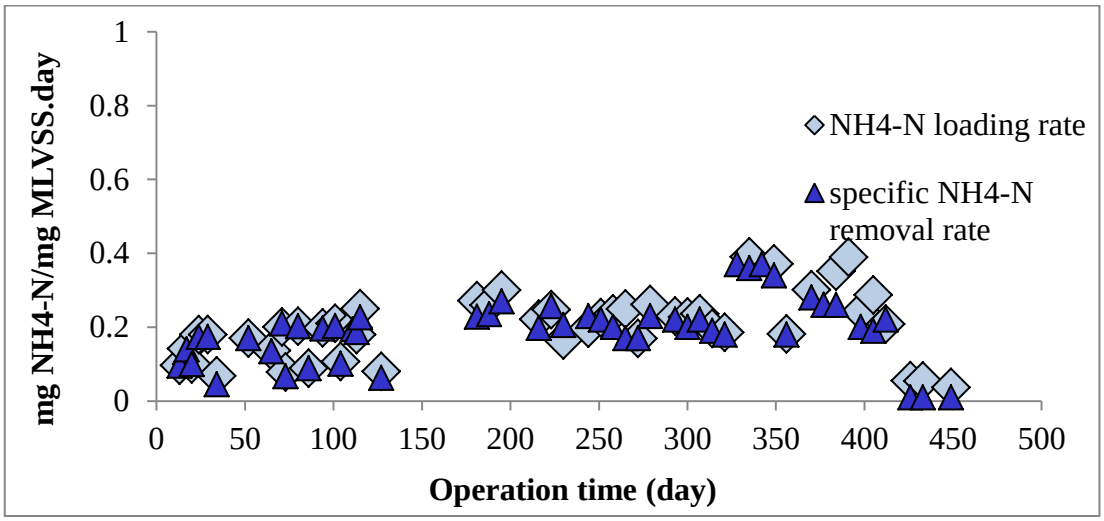


Figure 6.8. Ammonium loading and specific ammonium removal rates in R3 (Geyik and Çeçen, 2014a).

6.1.2.2. Effect of the COD/TKN ratio on EPS Production and Composition. Figure 6.9 shows the protein and carbohydrate content of EPS samples. Independent of the COD/TKN ratio, in all EPS fractions the protein content was higher. The average protein-EPS in reactors R1, R2 and R3 was about 19.5±2.8 mg/g MLVSS, 15.4±7.6 mg/g MLVSS and 18.1±6.8 mg/g MLVSS, respectively. Comparatively, the least protein-EPS was observed in R2 which received a lower organic loading than R1. At the time of EPS extractions, residual soluble COD in R2 varied in the range 15-80 mg/L while in R1 it was about 80-160 mg/L. Since organic matter dropped to low levels in R2, most probably, also some of the protein-EPS was consumed in this reactor. The consumption of protein- and carbohydrate-EPS in one running cycle is also stated in other studies (Zhu et al., 2012).

In general, the COD/TKN ratio seemed not to affect the production of total EPS to a great extent. An obvious effect was only seen in the case of carbohydrate-EPS in R3 sludge. In R3 sludge protein-EPS production was favoured while carbohydrate-EPS production (4.6±0.9 mg/g MLVSS) was obviously lower. In the absence of organic carbon in the feed, microorganisms could probably not afford to produce extracellular polysaccharides. Contrary to this, in R1 and R2 sludges, carbohydrate-EPS levels were higher (9.2±1.8 mg/g MLVSS, 8.6±2.4 mg/g MLVSS, respectively). Probably, in these reactors biomass could use organic carbon for cell synthesis while also affording production of extracellular polysaccharides. The relative amount of proteins and carbohydrates is important regarding the response of each sludge to toxic substances.

The total protein/carbohydrate (P/C) ratio in EPS (Figure 6.9-right y-axis) was calculated by taking into account the protein and carbohydrate content in all bound-EPS fractions, namely VLB-EPS, LB-EPS and TB-EPS. In literature studies, in activated sludge systems the P/C ratio is reported to vary in a wide range, from 0.1 mg/mg to 12.5 mg/mg, because of variable extraction methods and reactor operating conditions. In this study, P/C ratio varied between 1-13 mg/mg as shown in Figure 6.9

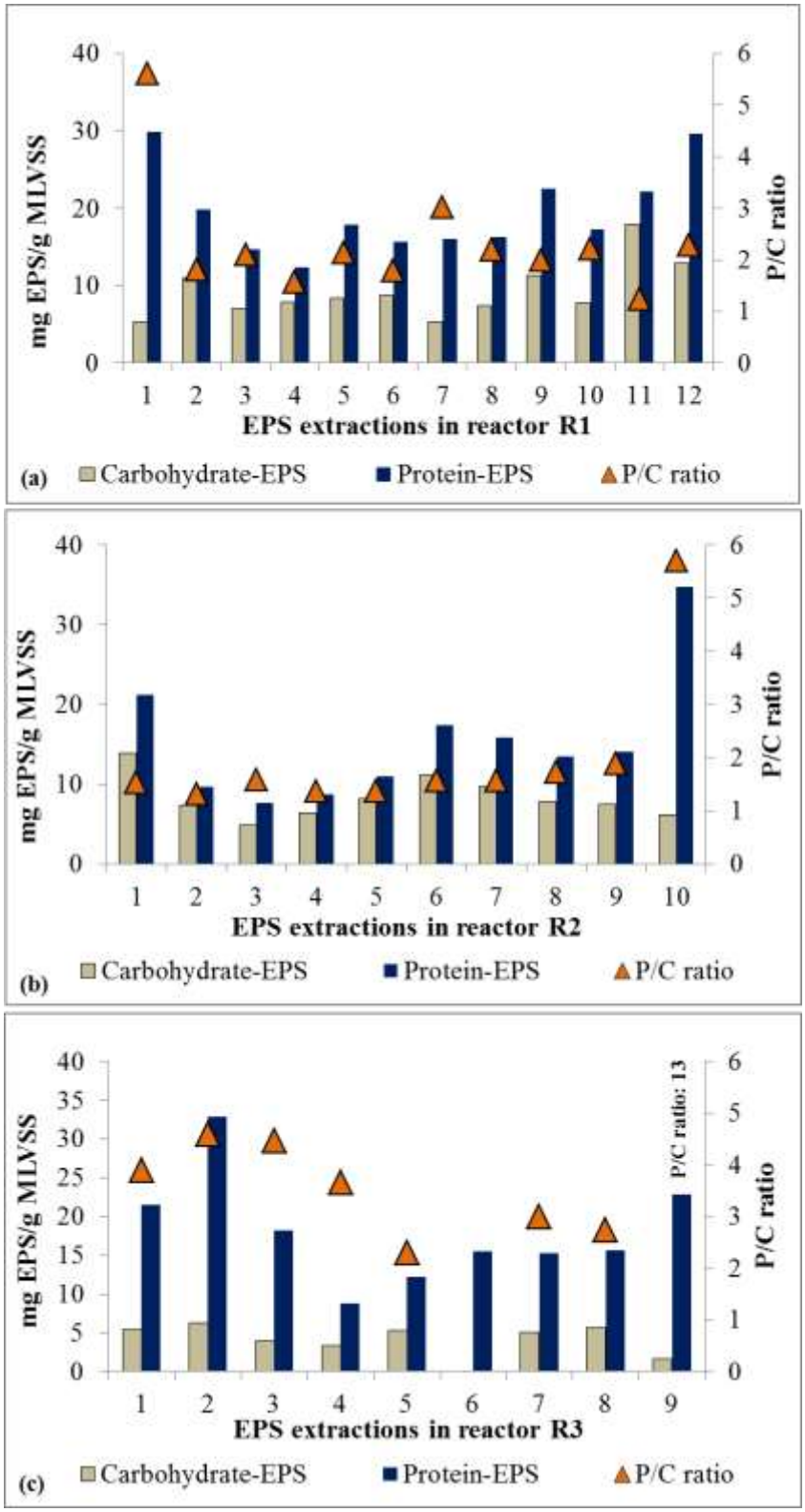


Figure 6.9. Composition of EPS in a) R1 (COD/TKN=10), b) R2 (COD/TKN= 5) and c) R3 (COD/TKN=0), left: Production of carbohydrate- and protein-EPS, right: Protein/Carbohydrate (P/C) ratios (Geyik and Çeçen, 2014a).

6.1.2.3. Fractionation of Bound-EPS. The bound EPS fractions shown in Figure 6.10, namely VLB-EPS, LB-EPS and TB-EPS, were calculated as the sum of carbohydrates and proteins. EPS might also be composed of other groups such as nucleic acids. However, the contribution of other groups is usually much smaller. In this part, EPS production was expressed in terms of EPS-mass (Figure 6.10, a1-a3) and COD of EPS (EPS-COD) per biomass (VSS) (Figure 6.10, b1-b3). In order to determine the latter, the COD of each EPS fraction was measured after extractions. Obviously, EPS-mass and COD of EPS paralleled each other.

As shown in Figure 6.10, total EPS production seemed to depend largely on TB-EPS whereas the production of VLB-EPS and LB-EPS was very low in all reactors (<4.5 mg/g MLVSS). Regardless of the COD/TKN ratio, the relative proportion of these fractions was almost the same in all reactors.

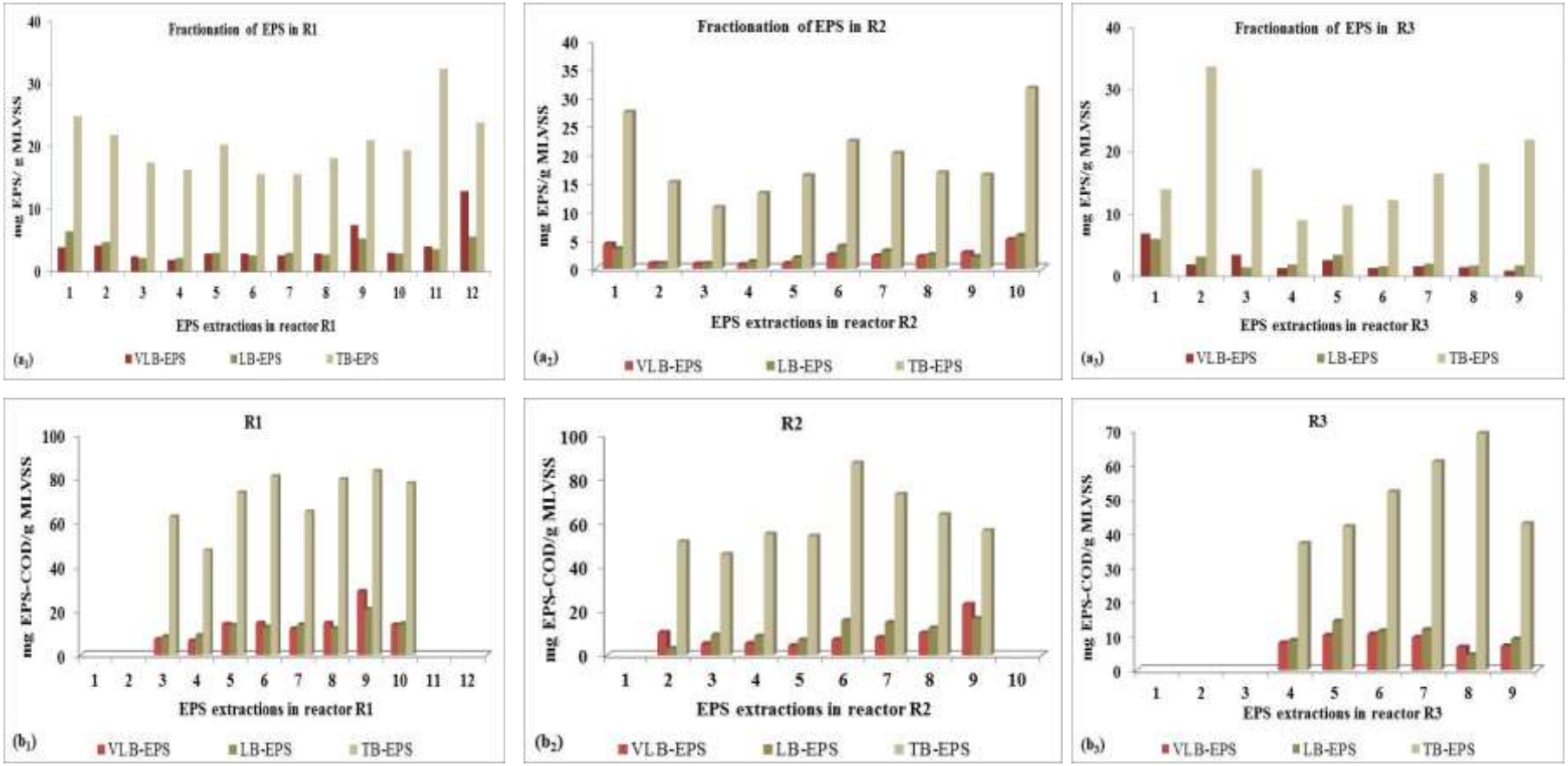


Figure 6.10. Expression of different bound EPS fractions (VLB-EPS, LB-EPS, TB-EPS) per MLVSS.

a_1, a_2, a_3 : EPS fractions in R1 (COD/TKN=10) , R2 (COD/TKN=5) and R3 (COD/TKN=0),

b_1, b_2, b_3 : The respective COD of each EPS fraction (Geyik and Çeçen, 2014a).

It is known from literature that the fraction of substrate that is not directed to energy production is used in active biomass synthesis and EPS production. One of the challenges is to correctly estimate the active heterotrophic yield Y_H in the presence of EPS formation (Ni and Yu, 2012). Respiration measurements (Çeçen and Kılıç, 2015) on R1 and R2 sludges revealed that about 60-50 % of substrate COD in R1 and R2 was directed to the energy reaction while the rest (40-50 %) was used in cell formation (Geyik and Çeçen, 2014a). Therefore, if the fraction of electrons shunted to EPS production is disregarded, Y_H should lie in the range of 0.4-0.5 mg cell COD/substrate COD. However, our calculations showed that bound EPS-COD constitutes about 3-10 % of total biomass COD. Thus, if both EPS production and active cell growth are taken into consideration, the real value of Y_H should be about 3-10 % lower. Most of EPS-COD, about 80 %, could be attributed to TB-EPS, while other fractions, VLB-EPS and LB-EPS, had much smaller contributions.

Another point examined was whether total EPS production, expressed as the sum of protein- and carbohydrate-EPS, or the formation of different fractions in EPS (very loosely bound, loosely bound, tightly bound) depended on substrate loading. For this purpose, total-EPS as well as its fractions were normalized by the initial substrate concentration. Contrary to expectations, total EPS production in R1 and R2 was not dependent on initial substrate concentration or organic loading rate. Even in R3 receiving no organic matter, total EPS production was at comparable levels as in R1 and R2 with dominant heterotrophic activity. Therefore, an important conclusion was that also a separate-stage nitrification system, as represented by reactor R3, had a high EPS production capacity although nitrifiers have a much lower growth rate and yield than heterotrophs (Geyik and Çeçen, 2014a).

6.1.2.4. Changes in the Molecular Weight of Protein-EPS.

Molecular Weight Distribution of Proteins in EPS. Figure 6.11 shows a typical distribution of proteins in the EPS of R1 sludge. The information presented in Table 6.1 bases on interpretation of about 250 fingerprints and shows the average distribution of proteins in each EPS fraction. In all EPS fractions low MW proteins within the range of 3.2-16.2 kDa were dominantly produced. In second place came the proteins with a MW between 19-74

kDa while only a small fraction consisted rather of high MW compounds with 2055≥MW≥ 1417 kDa. Generally, proteins in bound EPS fractions had a wide MW distribution.

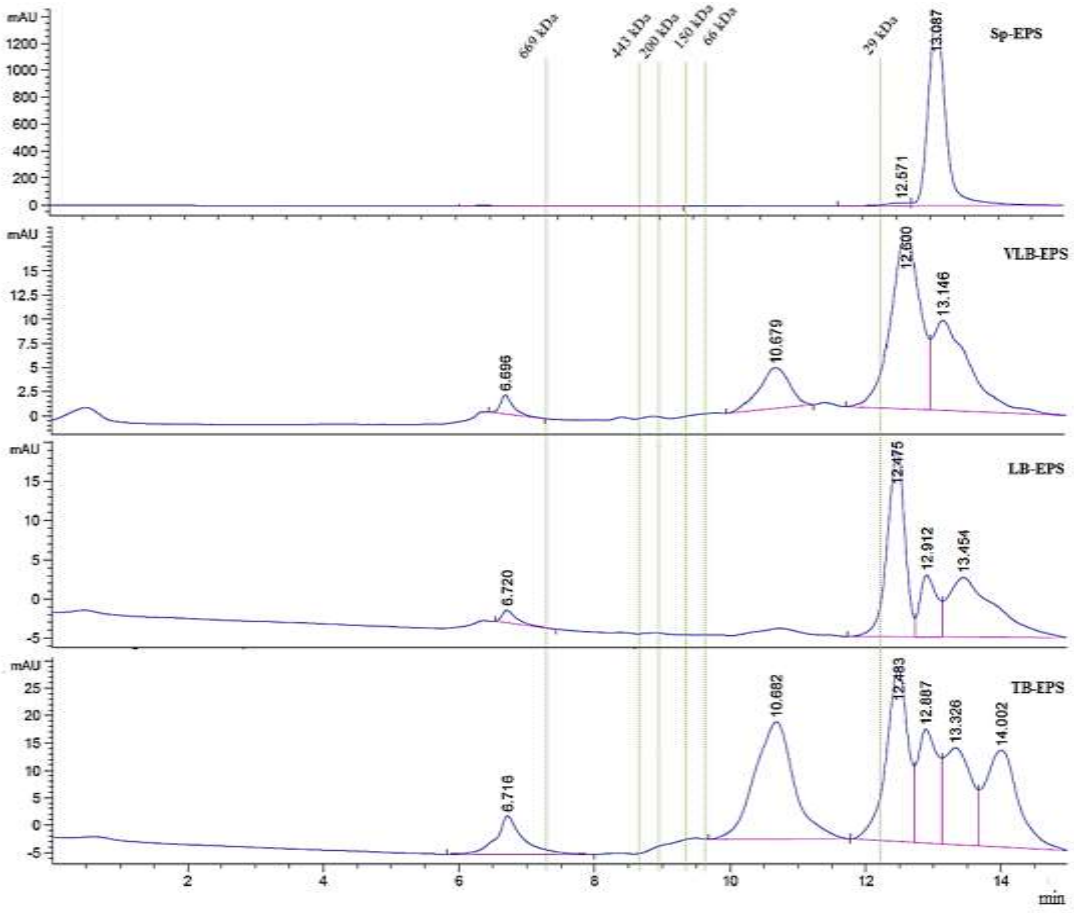


Figure 6.11. Typical chromatograms belonging to the different EPS fractions in R1 sludge. Sp: Supernatant-EPS, VLB-EPS:Very Loosely Bound-EPS, LB-EPS: Loosely Bound-EPS, TB-EPS: Tightly Bound-EPS.

Shifts of Proteins Among Different EPS Fractions. Wet analyses reported in Section 6.4.1 gave only a rough idea about the total protein content while detailed information could only be obtained from HPSEC chromatograms. Even small disturbances during reactor operation, such as a temporary decrease in dissolved oxygen concentration, led to changes in the structure of TB-EPS in R1 reactor that received a relatively high COD load. In such cases large proteins (≥1417 kDa) released from TB-EPS seemed to move to other fractions, in particular to the VLB-EPS fraction, to be then utilized by biomass. In R2 sludge receiving a lower organic loading, high MW proteins were observed in both TB-EPS and

Table 6.1. Molecular weight distribution of proteins in the different EPS fractions of R1, R2 and R3 sludges

Distribution of Proteins (%)					
EPS fractions	**Retention time (t) in min or molecular weight (MW) in kDa**				
	$6.3 \leq t \leq 6.7$	$t \leq 7.7$	$9.3 \leq t \leq 9.6$	$10.2 \leq t \leq 11.6$	$12.1 \leq t \leq 14.0$
	$2055 \geq MW \geq 1417$	**$MW \geq 641$**	**$150 \geq MW \geq 124$**	**$74 \geq MW \geq 19$**	**$16.2 \geq MW \geq 3.2$**
R1 (COD/TKN=10)					
Sp-EPS					100
VLB-EPS	5			8	87
LB-EPS					100
TB-EPS	5			11	84
R2 (COD/TKN=5)					
Sp-EPS					100
VLB-EPS					100
LB-EPS	7				93
TB-EPS	1		1	8	90
R3 (COD/TKN=0)					
Sp-EPS					100
VLB-EPS					100
LB-EPS					100
TB-EPS	1	1		11	87

LB-EPS fractions, but not in the VLB-EPS structure, indicating possibly their utilization by biomass. Also, according to wet protein analyses, R2 sludge produced the least protein-EPS.

In the EPS of R3 sludge, very large proteins were only observed in TB-EPS while in other fractions they were mostly below 16 kDa. In general, it is believed that shortage or complete absence of organic carbon, as in the case of R3, leads to secretion of proteins from TB-EPS fractions. These proteins are then most possibly consumed by biomass. Since VLB-EPS are very weakly bound, it is thought that they were initially consumed. Then came probably the consumption of LB-EPS in the absence or deficiency of organic carbon. It is believed that in the presence of toxic substances, movement of proteins will even increase under stress. Thus, toxics may move into receiving waters due to complexation with EPS-proteins.

HPSEC measurements gave also an insight into the type of proteins. Although proteins in all EPS fractions had similar MW, the responses of proteins, in other words their chromophoric properties, decreased with the COD/TKN following the order: R3>R2>R1. Upon the decrease of organic matter in the feed, EPS were observed to consist of proteins having higher chromophoric properties, indicating differentiation of proteins. In particular, in VLB and LB fractions of sludges the chromophoric properties of proteins changed with the COD/TKN ratio. It is most remarkable that proteins in the EPS of R3 sludge had higher chromophoric properties.

Before extraction of Sample No.9 (Figure 6.10a3), at about 430-450 days, R3 operation was disturbed due to an unknown reason and a very high protein content was measured by the wet method (Figure 6.9c). Probably, in this sample also intracellular proteins were measured and not only the proteins in EPS. On the other hand, HPSEC measurements could clarify the change in proteins. Chromatograms belonging to this period had very low absorbances, indicating a loss of protein-EPS.

Proteins in Supernatant Liquid. As seen in Table 6.1, proteins in supernatant EPS (Sp-EPS) were composed of low MW compounds with ≤16.2 kDa. HPSEC analysis confirmed that these proteins were not originating from peptone. The results led to the idea that protein-

EPS in the supernatant of all reactors (R1-R2-R3) have comparable characteristics. It was most remarkable that low MW proteins were also found in the supernatant of R3 sludge although this reactor was not fed with any organic substrate. Obviously, there was a release of proteins from bound EPS fractions as discussed above. In real systems, Sp-EPS play many roles such as complexation with metals.

Most possibly, the proteins recorded in Sp-EPS fall into the scope of substances called Soluble EPS (or SMP) which vary in a broad MW range (>600 kDa and <1 kDa). The unified EPS theory (Laspidou and Rittmann, 2002b) states that SMP can further be divided into two as Utilization Associated Products (UAP) and Biomass Associated Products (BAP), composed of low and high MW substances, respectively. In one literature work (Ni, 2013), under substrate-rich conditions, UAP having a MW lower than 250 kDa were the dominant type of SMP and were biodegraded during a run. Thereafter, in the endogenous phase, SMP with a higher MW of 4800 kDa were produced which were then regarded as BAP and constituted the dominant fraction in SMP. In our case, EPS extractions were mostly carried out on the day following feeding. Thus, the sludges were neither under feast nor famine conditions. According to our results, the supernatant seemed to be rather composed of UAP. The production of BAP with a relatively higher MW (1417 kDa) was more evident in bound EPS fractions (Table 6.1).

6.1.3. Comparison of EPS Production in Stabilization and Steady Periods

Table 6.2 compares the average level of EPS in the stabilization period and thereafter. However, examination of all data pointed to the need to consider the variations in EPS rather than the averages. For example, if average values were considered, in R1 sludge very similar EPS yields were recorded in both periods. However, the high standard deviation in the stabilization period was indicative of the high fluctuation in EPS. In R2 sludge, in the steady period protein-EPS was decreased approximately to 40 % of that in the stabilization period. However, in R3 sludge the production of EPS was similar in both periods, indicating a rapid stabilization in terms of amount and composition (Geyik and Çeçen, 2015).

Table 6.2. Comparison of EPS production in Reactor R1, R2 and R3 in the stabilization and steady periods.

Reactor	Characteristics of operation	EPS type	Stabilization period (mg/g MLVSS)	Steady period (mg/g MLVSS)	Comparison of periods
R1	Higher COD loading, COD/TKN:10 Heterotrophic and nitrifier activity (heterotrophic activity dominant)	Protein-EPS	22.3±8.4	19.5±2.8	No exact differences between the periods
		Carbohydrate-EPS	7.8±4.9	9.2±1.8	
		Total-EPS	30.2±11.0	28.5±4.4	
		P/C ratio	3.5±1.9	2.3±0.3	
R2	Limited COD loading, COD/TKN:5 Heterotrophic and nitrifier activity (nitrifier activity higher than in R1)	Protein-EPS	24.6±12.5	15.4±7.6	Gradual drop in protein-EPS in the stabilization period
		Carbohydrate-EPS	8.3±4.0	8.6±2.4	
		Total-EPS	32.9±15.0	23.6±8.6	
		P/C ratio	3.3±1.6	1.9±1.2	
R3	No COD loading, COD/TKN:0 Enrichment of sludge in nitrifiers	Protein-EPS	21.0±6.7	18.1±6.8	Rapid stabilization of EPS, similar yields in each period
		Carbohydrate-EPS	5.4±1.8	4.6±0.9	
		Total-EPS	26.4±6.6	22.2±7.3	
		P/C ratio	4.8±3.7	3.5±0.8	

6.2. Part II: Effect of Organic Carbon Source on EPS Production

This part aimed to clarify the role of organic carbon on the composition and fractionation of EPS. In literature, few studies show the relationship between substrate type and total EPS production. In addition, such studies have been mostly conducted in pure culture systems (Liu et al., 2010). Moreover, in most studies EPS changes are examined in the same reactor by exposing the sludge to different substrates. In such cases EPS production may be affected by former operational conditions. Ideally, to study the effect of substrate, several reactors should be run in parallel by keeping all parameters constant except the type of organic carbon. However, this procedure is adopted only in a limited number of studies (Ye et al., 2011; Zhu et al., 2012; Wang et al., 2013).

6.2.1. Monitoring Performances of the Reactors CR, RG and RP

In all reactors operated at a COD/TKN ratio of 10, loading rates were close to each other while only the type of organic substrate differed. In the main reactor, namely the control (CR) reactor, the average organic loading and specific substrate removal rates were about 0.17±0.04 and 0.15±0.03 mg COD/mg MLVSS.d, respectively. At the start of semi-continuous runs average COD was 961±72 mg/L and about 90% of it was removed. In RG, the glucose reactor, the average organic loading and removal rates were about 0.14±0.04 and 0.14±0.04 mg COD/mg MLVSS.d, respectively. Initial COD at the start of semi-continuous runs was 852±79 mg/L whereas about 94% removal was observed. In RP, the peptone reactor, the average organic loading and specific substrate removal rates were about 0.15±0.05 and 0.13±0.05 mg COD/mg MLVSS.d, respectively. The initial COD at the start of semi-continuous runs was 863±100 mg/L whereas about 91% removal was observed.

Respiration tests clearly indicated that in all activated sludge reactors the extent of organic carbon removal and nitrification was similar. In any case, organic carbon removal was more dominant than nitrification since the reactors were operated at the COD/TKN ratio of 10. The contribution of nitrification to total oxygen consumption was about 21%-37%. Detailed information about oxygen consumption can be found in a previous paper (Çeçen and Kılıç, 2015).

6.2.2. Hydrophobicities and Surface Charges of Different Sludges

In parallel to EPS extractions, surface charge and hydrophobicity were weekly measured. The results were interpreted along with EPS extractions.

Figure 6.12a shows the hydrophobicity of different sludges. The hydrophobicity of CR and of RG sludges were not statistically different from each other (p<0.05), as determined by the t-test at 95 % confidence level. On the other hand, hydrophobicities of RG and of RP (p<0.05) as well as CR and RP (p<0.05) were significantly different from each other (Table 6.3).

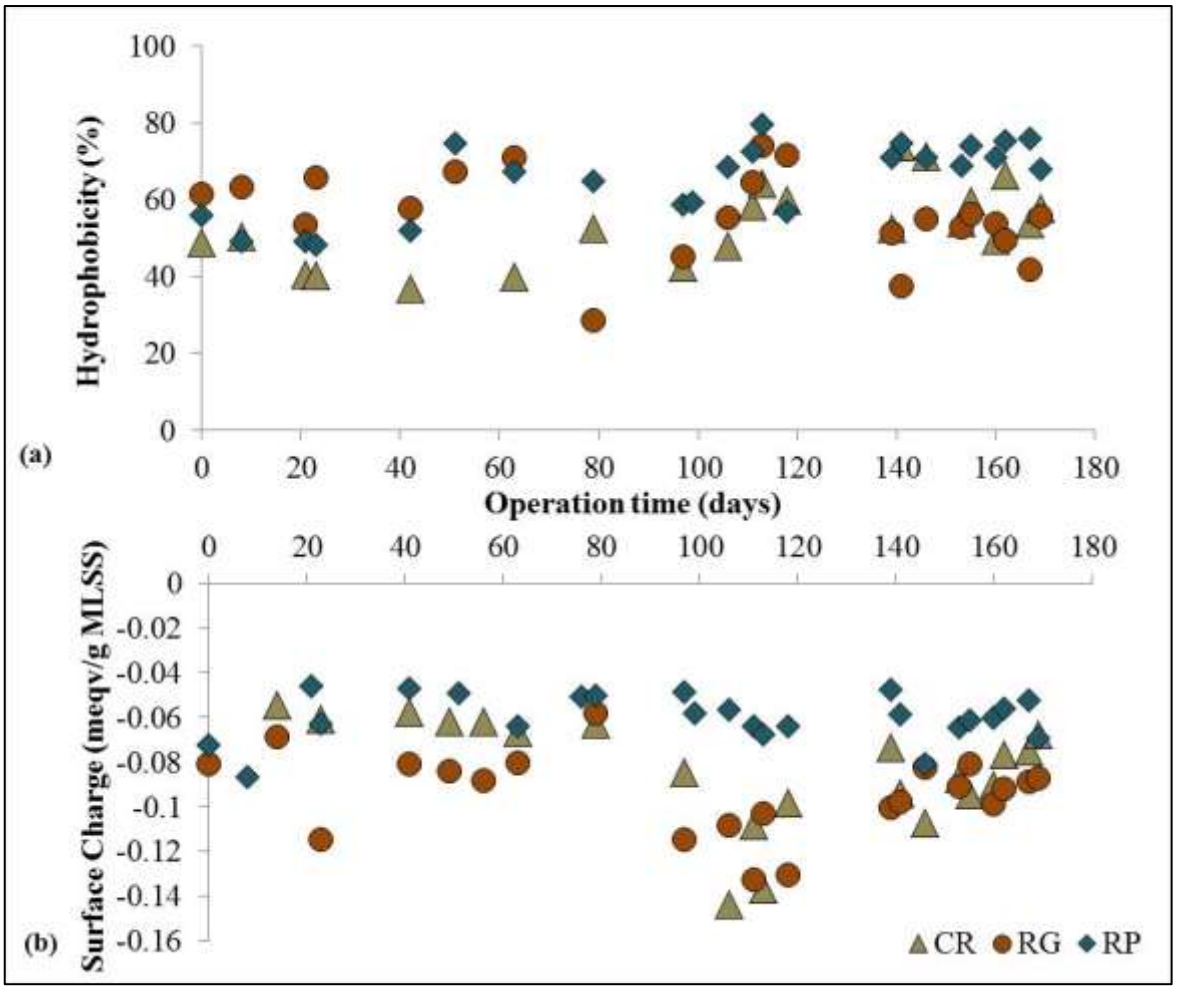


Figure 6.12. a) Hydrophobicities and b) Surface charges of sludges taken from Control (CR), Glucose (RG) and Peptone (RP) reactors (Geyik et al., in press).

Table 6.3 shows the average surface charges in CR, RG and RP sludges. Statistical analysis by the t-test indicated that surface charges in CR and RG sludges were not significantly different (p<0.05) from each other at 95 % confidence level. On the other hand, RP sludge had a less negative surface charge. The surface charge of RG and RP sludges (p<0.05) as well as CR and RP (p<0.05) were significantly different from each other.

Table 6.3. Average hydrophobicities and surface charges of different sludges.

Sludge	**Hydrophobicity (%)**	**Surface Charge (meqv/g MLSS)**
CR (Contol Reactor)	53±10	-0.084±0.02
RG (Glucose Reactor)	56±11	-0.094±0.02
RP (Peptone Reactor)	65±9	-0.060±0.01

Thus, the type of substrate seemed to influence hydrophobicity and surface charge. In a sludge the hydrophobic fraction is reported to consist of proteins (Durmaz and Sanin, 2003). In our case RP sludge fed with peptone (a mixed substrate largely consisting of unidentified proteins) had the highest hydrophobicity. In CR sludge peptone was present only in small amounts while no peptone was present in RG (Table 5.1). Under protein deficiency, these two sludges had lower hydrophobicities than RP. According to some studies, the negative surface charge can result from the carbohydrates in EPS (Liao et al., 2001). In RG and CR, the amount of glucose was relatively high. Therefore, these two sludges had a higher negative surface charge than that of RP. Confirming most literature data, also in the present study, a negative correlation was observed between surface charge and hydrophobicity as shown in Figure 6.13.

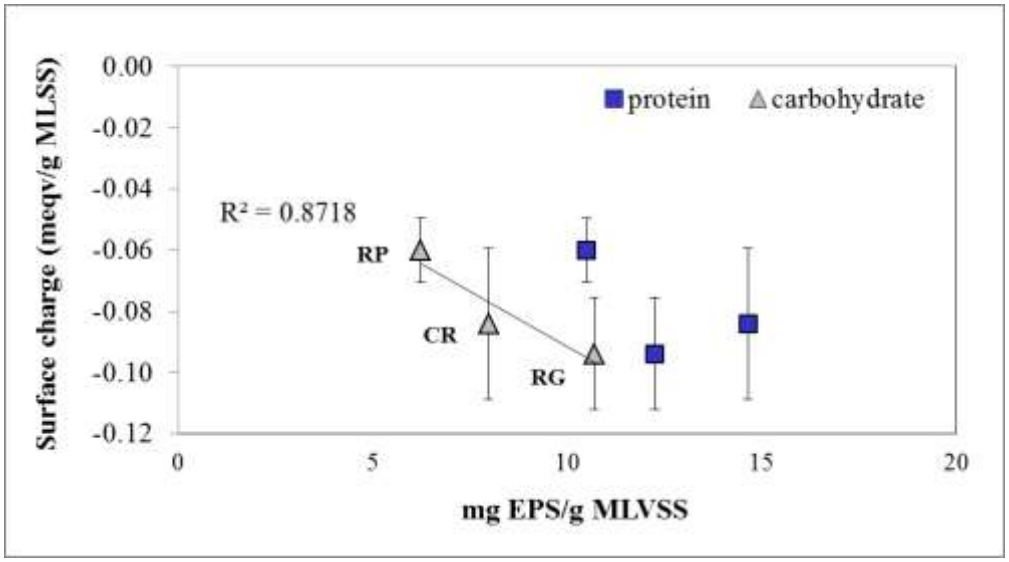


Figure 6.13. Relationship between protein- and carbohydrate-EPS and surface charges of activated sludges (CR: Control Reactor; RG: Glucose Reactor; RP: Peptone Reactor) (Geyik et al., in press).

6.2.3. Production of Protein and Carbohydrate in Bound-EPS

Figure 6.14a-c presents the protein and carbohydrate content of EPS extracted from CR, RG and RP sludges. Independent of substrate type, in each sludge protein dominated and determined the total level of EPS. Statistical analysis by the t-test indicated that the protein content in the EPS of the RP sludge was statistically different from the RG sludge ($p=0.012$). Protein-EPS in CR, RG and RP were about 14.6±3.8 mg/g MLVSS, 12.3±2.6 mg/g MLVSS and 10.5±4.4 mg/g MLVSS, respectively.

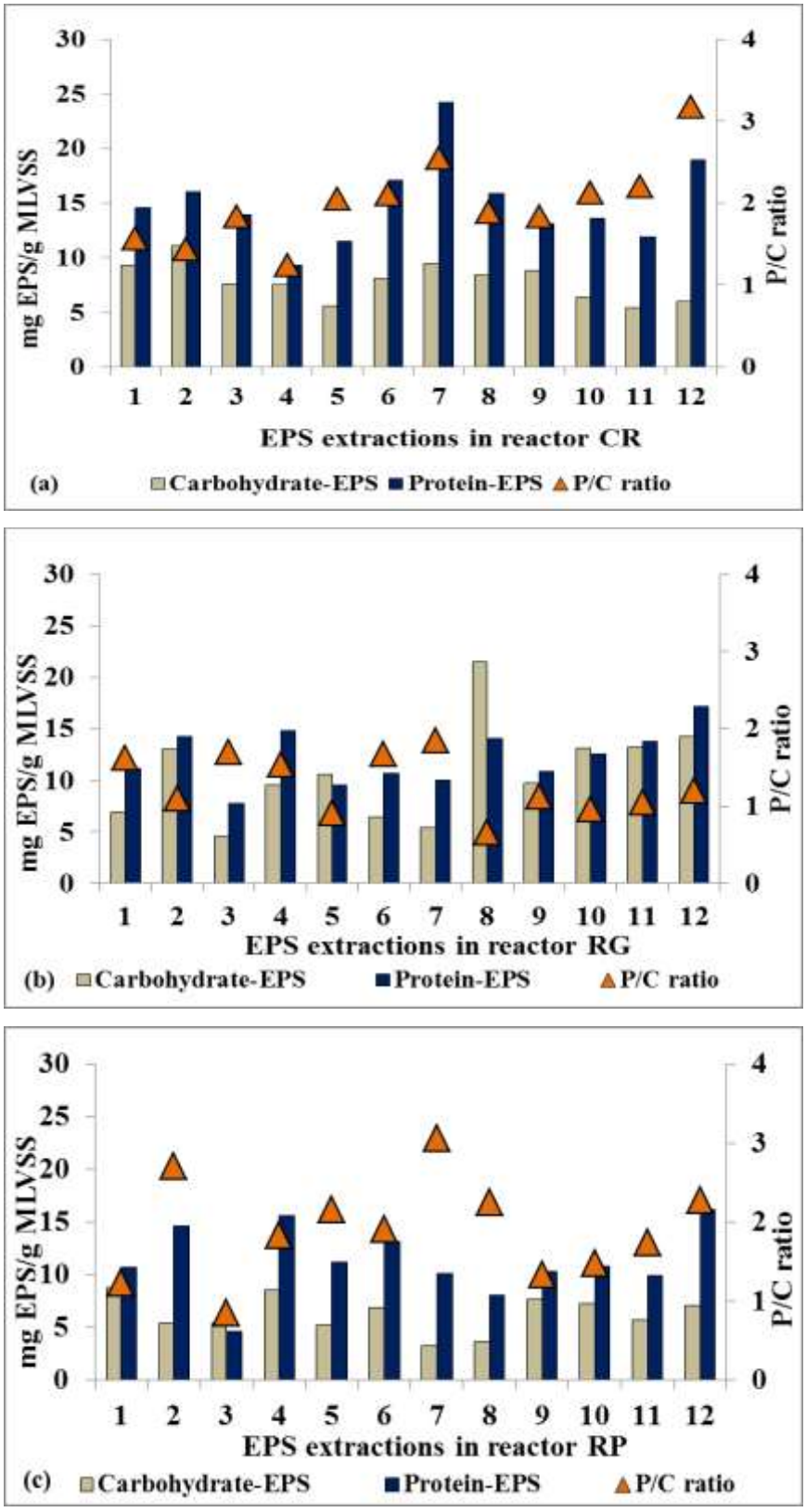


Figure 6.14. Production of protein- and carbohydrate-EPS in a) Control (CR), b) Glucose (RG) and c) Peptone (RP) sludges (Geyik et al., in press).

Statistical analysis by ANOVA indicated that the carbohydrate content in EPS was significantly different in each sludge (p<0.005). Analysis of data in Figure 6.14a-c showed that carbohydrate-EPS production followed the order RG>CR>RP. In RG carbohydrate-EPS production (11.8±4.3 mg/g MLVSS) was obviously more favoured than in others. Moreover, in terms of negativity, surface charge followed the same order as indicated above. These results suggest that in real biological treatment systems the protein and carbohydrate content of EPS would highly depend on the type of substrate in a wastewater.

The ratio of protein to carbohydrate (P/C) in EPS is shown in Figure 6.14 (right y-axis). In literature, in activated sludge systems this ratio varies in a wide range. This is likely to result from the differences in extraction methods as well as reactor operation. As calculated from the data in Figure 6.14a-c, the average P/C ratio in CR sludge was about 1.9. In the RP sludge it was about 2.1 while in RG it was clearly lower, at about 1.3.

6.2.4. Examination of the Different Fractions in Bound-EPS

As shown in Figure 6.15, compared with TB-EPS, production of VLB-EPS and LB-EPS was very low in all sludges (<6 mg/g MLVSS). Therefore, TB-EPS are of greater importance since total-EPS largely depends on this fraction. This observation is in agreement with literature findings (Bezawada et al., 2013; Tseng et al., 2015).

During adaptation to new feeds, in RG and RP sludges production of VLB-EPS and LB-EPS was not much affected. These fractions were already produced at low levels (<6 mg/g MLVSS) in all reactors and small fluctuations were not noticable. On the other hand, TB-EPS production was much higher and relatively higher fluctuations were observed. This resulted in differences in total-EPS. Such differences in TB-EPS were particularly noticable in CR and RG sludges which contained glucose.

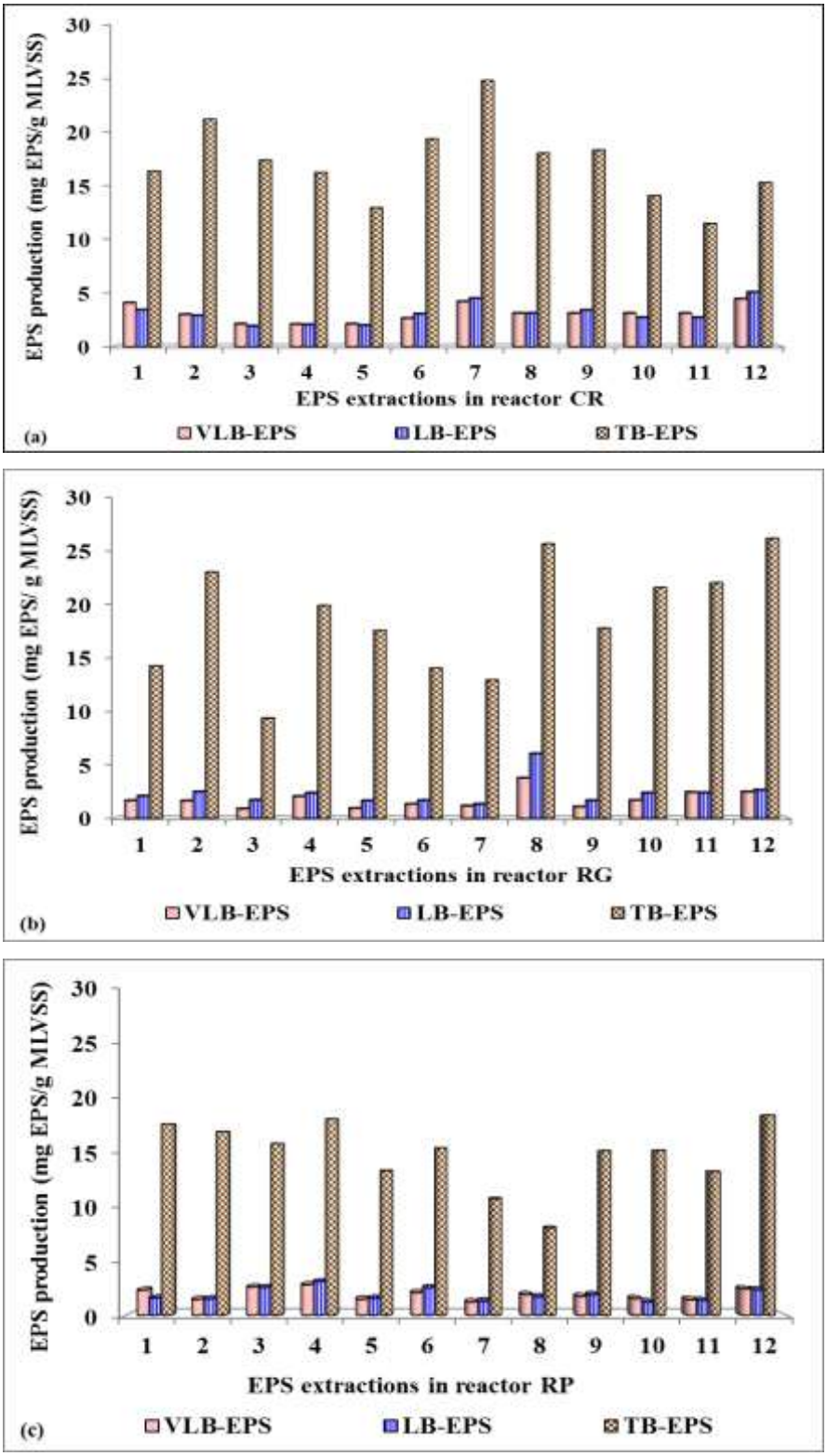


Figure 6.15. Fractionation of bound EPS into Very Loosely Bound (VLB-EPS), Loosely Bound (LB-EPS) and Tightly Bound (TB-EPS) fractions in a) CR, b) RG and c) RP sludges (Geyik et al., in press).

As shown in Figure 6.15b, in RG it took a longer time for TB-EPS to come to a stable level, probably due to variable biodegradation rates of glucose as reported in literature (Wei et al., 2008). In this sludge TB-EPS varied in the range of 10-25 mg/g MLVSS with an average value of 18.8±5.3 mg/g MLVSS. The coefficient of variation was relatively high (~30 %). On the other hand, RP sludge adapted very soon to peptone feeding, as shown from the more stable EPS production at the beginning (Figure 6.15c). In this sludge TB-EPS varied in the range of 15-25 mg/g MLVSS while the average was 15.0±3.1 mg/g MLVSS. The coefficient of variation was at an acceptable level (20 %) for biological systems (Reed et al., 2002). The importance of substrate type for EPS yield is also indicated in literature studies (Yu et al., 2008; Wang et al., 2014).

6.2.4.1. Total Level of EPS Production. In our case, in the activated sludges CR (control) and RG (glucose), total-EPS were higher than in RP (peptone) although carbon removal and nitrification efficiencies of all reactors were comparable. Peptone is a mixed and complex substrate consisting of animal products. These characteristics might have led to a lower EPS production compared to CR and RG sludges that were fed with relatively simpler organic substrates.

In literature, usually EPS are roughly fractionated while few studies examine all EPS fractions (Ye et al., 2011a). In the present study total-EPS (total bar width) were divided into the fractions shown in Figure 6.16.

VLB-EPS and LB-EPS fractions averaged about 8-13 % of total-EPS while the rest consisted of TB-EPS. As seen in Figure 6.16, in RP sludge total-EPS was slightly lower than in others. However, in this sludge the relative weight of each EPS fraction was similar to other sludges. Thus, substrate type did not appear to affect fractionation while it seemed to have a substantial effect on composition of EPS.

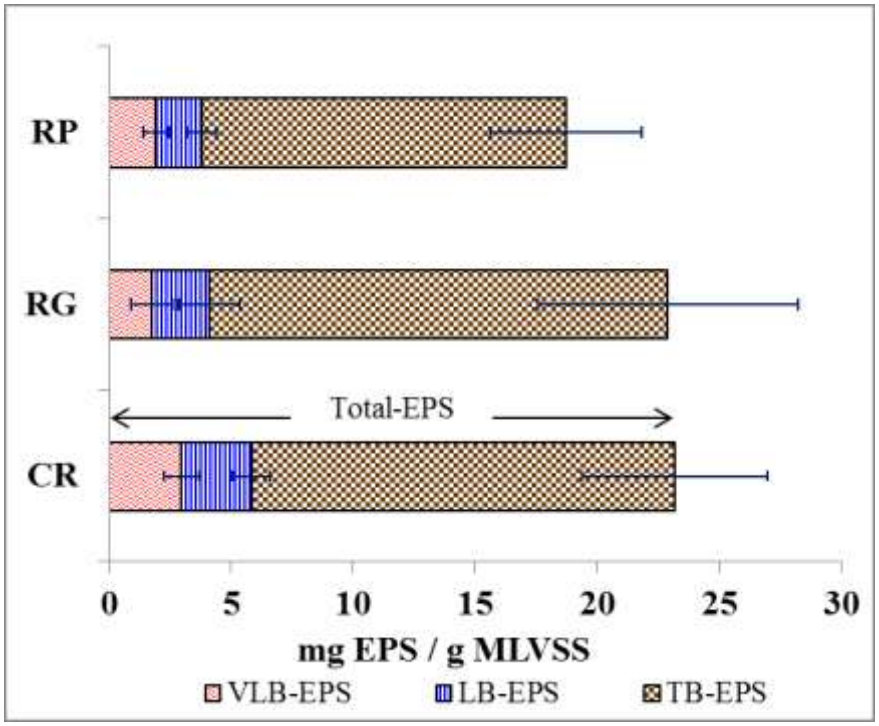


Figure 6.16. Fractionation of bound-EPS in CR (Control), RG (Glucose) and RP (Peptone) sludges: Very Loosely Bound (VLB-EPS), Loosely Bound (LB-EPS) and Tightly Bound (TB-EPS) fractions (Geyik et al., in press).

6.2.5. Examination of Protein-EPS by HPSEC

The use of HPSEC allowed the identification of the change in protein-EPS upon applying a new feeding regime to each sludge. As observed from absorbance values, in each sludge proteins had different chromophoric properties which decreased in the order of RP>>CR>RG. These differences are thought to be related with the organic composition of feeds.

In CR sludge which was started up first, EPS were already stabilized. This sludge was then divided into two reactors (RG and RP) to examine the effect of new feeds on EPS. When glucose was fed to the RG reactor (Feed G in Table 5.1), in the first two extracts EPS chromatograms were very similar to the original sludge CR fed with Feed 1. Thus, using this feed rich in carbohydrate instead of the balanced Feed 1 did not bring about a dramatic change in protein-EPS until about 10 days. However, continuation of glucose feeding led to a reduction in the MW variety of proteins. Probably, protein in EPS

was also deteriorated since splitted chromatograms were observed. Yet, after four months of operation, proteins that had once disappeared were seen to reemerge in the VLB-EPS and LB-EPS fractions of RG sludge and fingerprints stabilized. On the other hand, the quantity of protein lost from TB-EPS was not recovered to the same extent.

Protein loss in TB-EPS is an important point to consider since the essential fraction of EPS is TB-EPS. If in long-term operation the sludge has a low amount of proteins, wastewater microorganisms might be very susceptible to toxic substances such as heavy metals since protein-EPS have the ability to complex with metals. Some studies indicated that sorption of heavy metals can protect microorganisms from toxic effects (Decho, 2000) and proteins have a high binding strength and affinity to metals (Pan et al., 2010).

Contrary to RG, the RP sludge did not pass through an adaptation period when the feed shifted to a more proteinaceous one (Feed P in Table 5.1) than that used for CR. As the reactor run continued, even new protein with different MW emerged in EPS chromatograms, particularly in the TB-EPS fraction. While the MW variety of protein was about 3-5 in the CR and RG sludge, in RP sludge this increased up to 8 as shown in Figure 6.17.

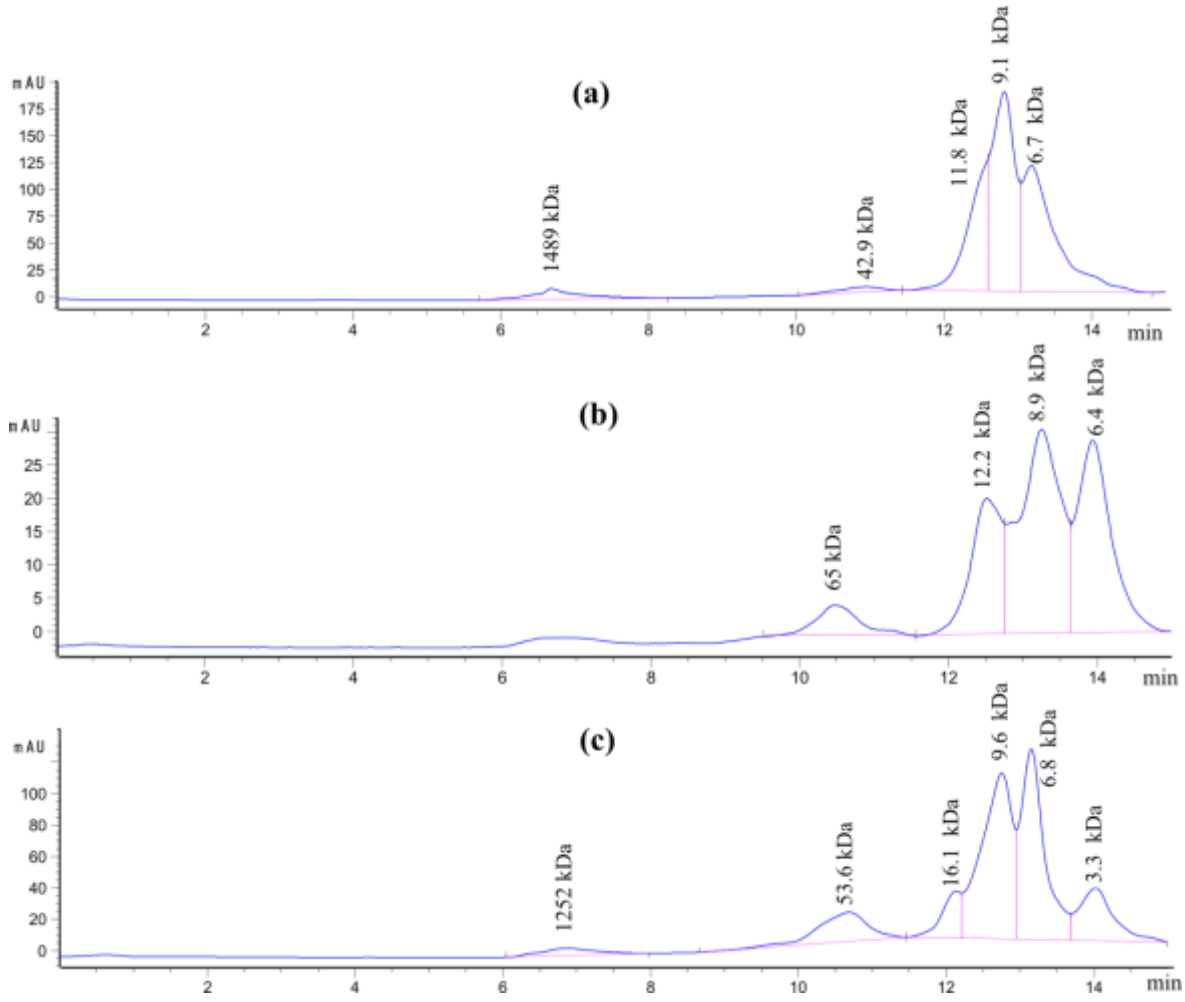


Figure 6.17. Typical HPSEC fingerprints of TB-EPS.

a) Control (CR) sludge b) Glucose (RG) sludge c) Peptone (RP) sludge.

(Sp-EPS: Supernatant-EPS, VLB-EPS: Very Loosely bound-EPS, LB-EPS: Loosely Bound-EPS, TB-EPS: Tightly Bound-EPS).

6.2.5.1. Determination of Molecular Weight (MW) Distribution in Protein-EPS. Table 6.4 represents the evaluation of about 300 fingerprints in terms of MW distribution in each bound EPS fraction as well as in supernatant-EPS. As seen in this table, in all sludges low MW (3.3≤MW≤13.6 kDa) proteins were dominant. The type of organic substrate seemed to influence the sizes of both bound and soluble proteins.

Table 6.4. Characterization of protein-EPS in terms of MWs in CR, RG and RP sludge (average values).

MW range of proteins in kDa	% distribution of protein-EPS in CR, RG and RP sludges											
	Sp-EPS			**VLB-EPS**			**LB-EPS**			**TB-EPS**		
	CR	RG	RP	CR	RG	RP	CR	RG	RP	CR	RG	RP
1252 ≤ EPS ≤ 2280				6		7	10		15	10	3	5
28 ≤ EPS ≤ 46				10								
40 ≤ EPS ≤ 66										22	5	10
15.9 ≤ EPS ≤ 22.7												4
7.3 ≤ EPS ≤ 13.6	100	18	1	19	40	10	40	56	22	44	60	33
3.3 ≤ EPS ≤ 7.3		82	99	65	60	83	50	44	63	24	32	48

Sp-EPS: Supernatant-EPS (Sp-EPS); **VLB-EPS:** Very Loosely Bound EPS**; LB-EPS:** Loosely Bound EPS; **TB-EPS:** Tightly Bound EPS. **CR:** Control Reactor, **RG:** Glucose Reactor, **RP:** Peptone Reactor.

Compared to other sludges, in RP a large portion of proteins had very small MWs while only a small portion consisted of high MW compounds (1252≤MW≤2280 kDa). Whenever high MW proteins were observed in the TB-EPS fraction, the same were concurrently detected in other fractions. For example, as shown in Table 6.4, in CR and RP sludges large proteins (1252 ≤ MW ≤ 2280 kDa) seen in TB-EPS appeared also in other bound fractions. This pointed to a gradual shift of proteins from tightly bound EPS to loosely bound ones. Generally, a wider MW distribution was seen in the TB-EPS fraction.

In RG sludge only a small percentage of proteins had a high MW. However, they were not concurrently detected in other fractions. RG sludge was fed with glucose alone, thus it was deficient in proteins. Most probably, under such conditions, high MW proteins released from TB-EPS moved to loosely bound fractions (VLB-EPS and LB-EPS) to be then utilized. As indicated in literature, in the case of protein deficiency, microorganisms can potentially degrade EPS components. Small molecules produced as a result of this degradation might then be used as a source of carbon and energy (More et al., 2014).

As seen in Table 6.4, in all sludges proteins in supernatant EPS (Sp-EPS) were composed of low MW compounds with ≤13.6 kDa. Particularly, in Sp-EPS of RP almost all proteins had very small sizes. HPSEC analyses confirmed that these small proteins were not originating from peptone itself. Therefore, they might be considered within the scope of substances called Soluble EPS (or SMP) (Laspidou and Rittmann, 2002). Such proteins in Sp-EPS are likely to form complexes if the sludge is exposed to heavy metal ions such as Ag^{+} (Geyik and Çeçen, 2014b).

6.2.6. Combined Evaluation of EPS, Hydrophobicity and Surface Charge

Colorimetric results were interpreted along with HPSEC data. Although the absolute amount of protein-EPS was smaller in RP sludge, the highest P/C ratio was observed in this sludge (Figure 6.18). Despite the fact that the CR sludge had a higher amount of protein-EPS, this sludge had a lower hydrophobicity since the P/C ratio was smaller, indicating the dominance of carbohydrate. Therefore, the absolute amount of protein-EPS is not as relevant as the EPS P/C ratio (Geyik et al., in press).

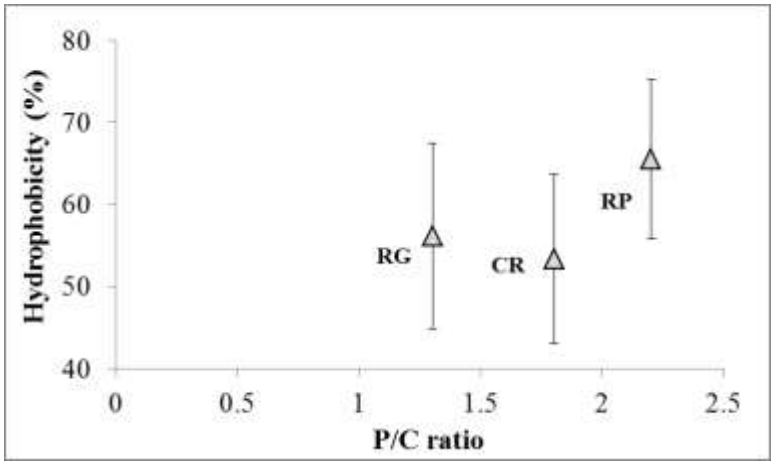


Figure 6.18. Relationship between protein/carbohydrate (P/C) ratio and surface hydrophobicity of activated sludges (Geyik et al., in press).

As shown before in Figure 6.13, an increase in the carbohydrate content of EPS correlated with more negative sludge surface. RP sludge had the least amount of carbohydrate in this sludge, EPS had also the lowest negativity. However, RG and CR sludges were fed with carbohydrate whereas a higher amount was fed to the former. Due to high production of carbohydrate-EPS in each case, the surfaces of these sludges became more negative. These results are in agreement with literature studies (Higgins and Novak 1997; Shin et al. 2000; Liu and Fang 2003; Hoa et al. 2003; More et al. 2014).

6.3. Part III: Effect of Silver (Ag^+) and Nanosilver (AgNP) on EPS Production

This part was divided into two sections as

(i) short-term experiments

(ii) long-term experiments.

Short-term experiments aimed to determine inhibitory effects of Ag^+ and AgNP on CR sludge that was fed with Feed 1 resembling domestic wastewater. As indicated in Section 3.10.2, Ag^+ and AgNP are used in many consumer products due to their antimicrobial properties. Therefore, they can easily be released into domestic wastewater treatment plants. In order to determine the inhibitory level of Ag^+ and AgNP, short-term respirometric tests were first conducted with activated sludge samples. In long-term

experiments in semi-continuously fed batch reactors, AgNP was added to reactor CR and RP as indicated in Section 5.1.3.

6.3.1. Type of AgNP Used in Experiments

Initially, experiments were started with the commercially available AgNP (Sigma-Aldrich). It was purchased as a powder and was not coated with any organics. The particle size was < 100 nm as declared by the company. When a solution was prepared by using this powder, the suspension was turbid and had a dark grey color as shown in Figure 6.19a. On the other hand, the synthesized AgNP solution was yellow colored (Figure 6.19b).

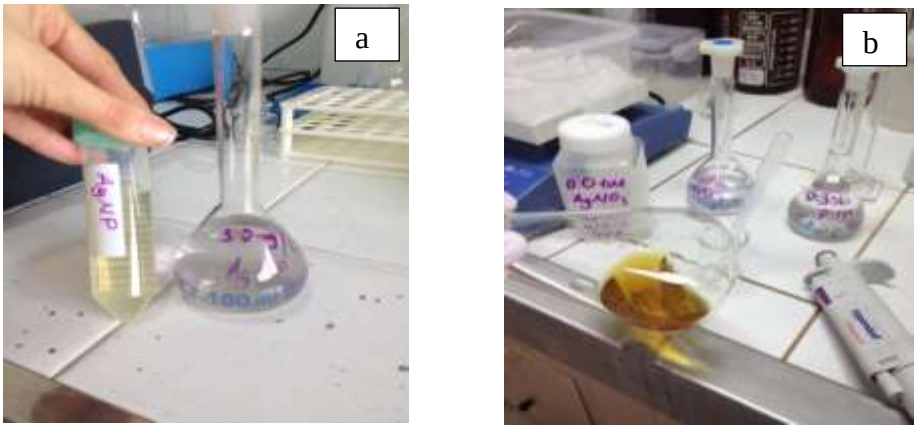


Figure 6.19. Types of nanosilver a) Powder AgNP, b) Synthesized yellow-colored AgNP.

Both solutions were scanned in the range of 300-700 nm using a UV–vis spectrophotometer to obtain a sharp peak at 400 nm which is an indicator of AgNP formation. In the visible spectrum range, commercial AgNP solution (Figure 6.20b) did not exhibit a typical AgNP spectrum since particles clumped together to form aggregates that were easily detectable by eye. On the other hand, a sharp peak located at 400 nm was obtained while recording synthesized AgNP. Both solutions had the same AgNP concentration while recorded spectrums were different as shown in Figure 6.20.

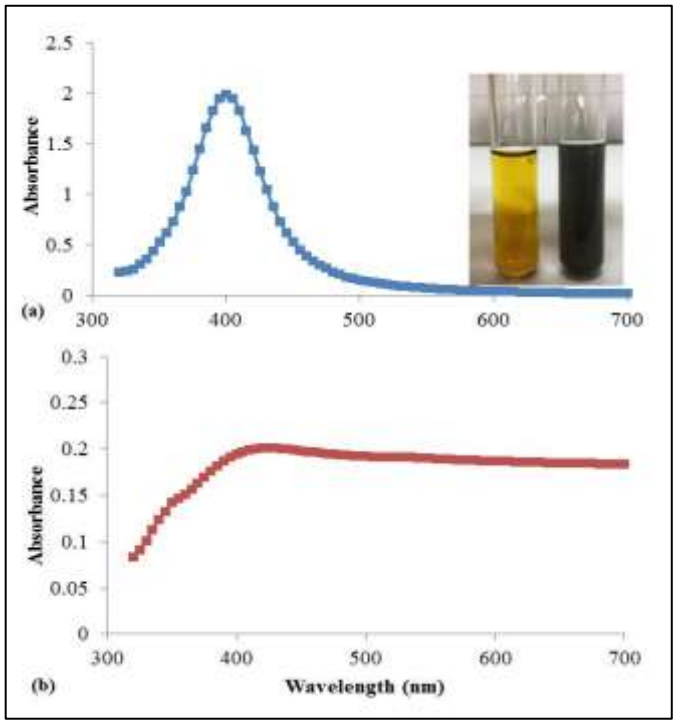


Figure 6.20. a) UV-Vis absorption spectrum of clear yellow colloidal synthesized AgNP, (inside) Visual difference between nanosilver stock solutions (the yellow colloidal AgNP was synthesized by reduction with $NaBH_4$, the dark grey solution was prepared by using AgNP powder), b) UV-Vis absorption spectrum of dark grey commercial AgNP.

The stability of synthesized AgNP was monitored for two months by measuring the spectrum in 10 days intervals. It was seen that AgNP sustained their stability over two months (Figure 6.21). The hydrodynamic diameter of synthesized AgNP was also measured; the average size was 38±10 nm. The zeta potential of the clear yellow AgNP solution was -25.55±0.88 mV. However, in the case of commercially available AgNP these two parameters could not measured due to settling of particles during measurement.

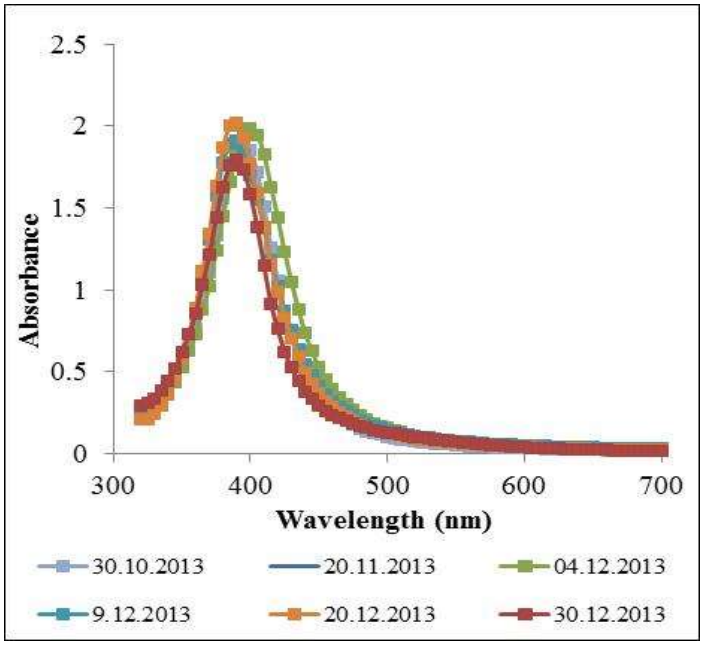


Figure 6.21. Changes in the spectrum of synthesized AgNP with respect to time.

The particles were also visualized by Environmental Scanning Microscopy (ESEM). The images showed that particles in commercial AgNP were clamped together and had different sizes. On the other hand, synthesized AgNP was dispersed in the PVP medium and particles had a size < 10 nm as seen in ESEM analysis (Figure 6.22).

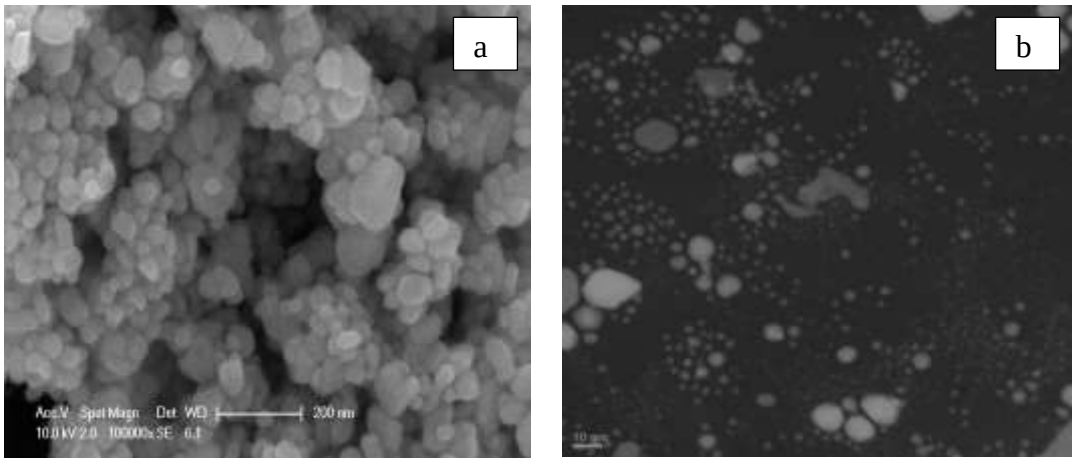


Figure 6.22. ESEM images: a) commercial AgNP and b) synthesized AgNP.

6.3.2. Short-Term Exposure of Activated Sludge to Ag^+ and AgNP

Activated sludge respiration tests were initially carried out by using commercially available AgNP (Sigma-Aldrich). In respirometric chambers, 0-15 mg/L AgNP was added along with necessary nutrients. O_2 consumption was the same in control and AgNP added chambers. In those chambers, cumulative O_2 consumption reached approximately 50 mg O_2 in 24 h during organic matter removal and nitrification. In these tests, 15 mg/L AgNP did not exert any inhibitory effect as shown in Figure 6.23. Most probably, commercial available AgNP was present in metallic form (Ag^0) and did not create inhibition. On the other hand, 63 % inhibition of cumulative oxygen consumption was observed in the presence of 3 mg/L of Ag^+.

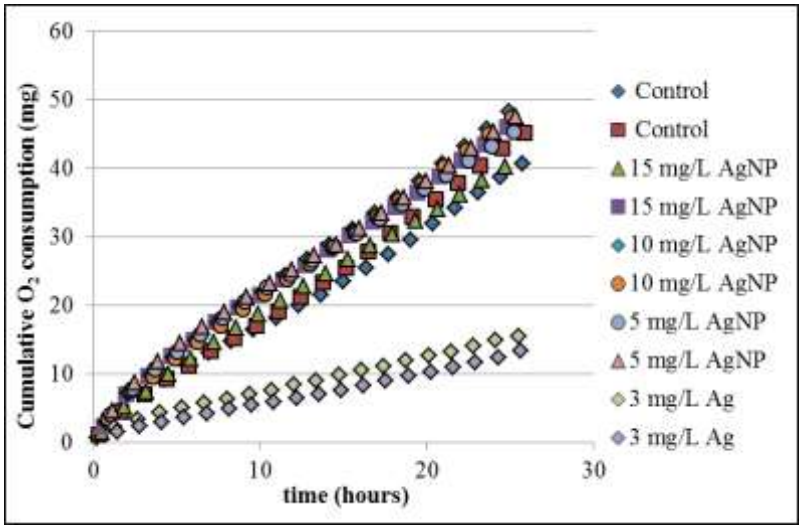


Figure 6.23. Cumulative O_2 consumption of activated sludge in the presence of commercial Ag^+ and AgNP.

Experiments were then continued with the AgNP synthesized in laboratory. First, the possible toxicity of materials used in synthesis, namely PVP and $NaBH_4$, was also checked by respirometric tests. The concentrations of PVP and $NaBH_4$ were kept at the same level as in AgNP synthesis. As seen in Figure 6.24, in control chambers as well as PVP and $NaBH_4$ added chambers, O_2 consumption was the same, indicating that these two materials did not exert any toxic effect on sludge.

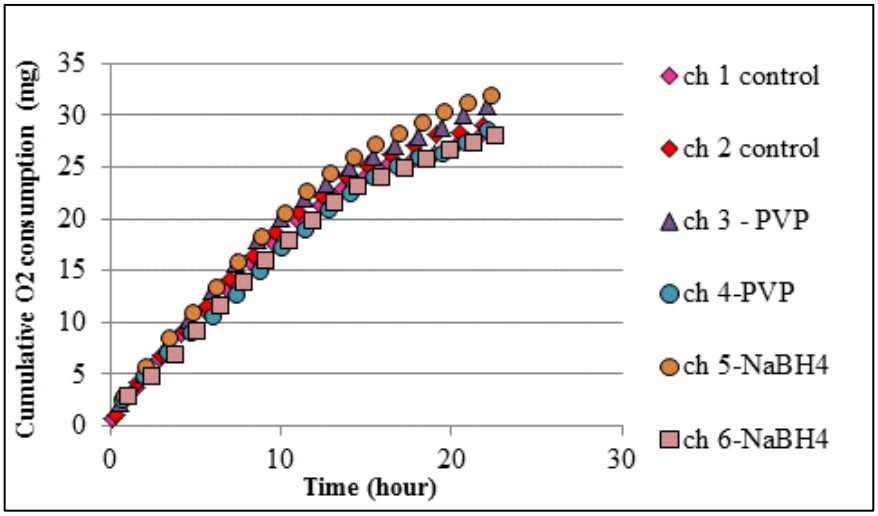


Figure 6.24. Effect of PVP and $NaBH_4$ on the cumulative O_2 consumption in activated sludge.

As seen in Figure 6.25, 0.5-2 mg/L AgNP had no significant inhibitory effect on sludge respiration; O_2 consumption was similar in control and AgNP-added chambers. On the other hand, 4 mg/L AgNP inhibited O_2 consumption by 25 %. Considering the result of previous theses (Ayyıldız, 2013; Kılıç, 2014), this slight decrease at 4 mg/L AgNP was attributed to inhibition of nitrification, and not organic carbon removal.

On the other hand, as seen in Figure 6.23 and 6.25, 3-4 mg/L Ag^+ was much more inhibitory than AgNP at the same dose. This level of Ag^+ caused complete inhibition of nitrification whereas it led to some decrease in organic carbon removal (Ayyıldız, 2013; Kılıç, 2014; Çeçen and Kılıç, 2015). When results were evaluated by using Graph Pad Prizm 6 for Windows, nonlinear fitting indicated that the IC_{50} value for Ag^+ ranged between 2.3-3.0 mg/L (R^2=0.75-0.95) when the sludge was exposed to Ag^+ for 21 hours. On the other hand, the IC_{50} value for AgNP ranged between 3.2-11.1 mg/L (R^2=0.52-0.92) after 21 hour contact. Thus, the IC_{50} value of AgNP was considerably higher compared to Ag^+ ion. This showed that AgNP was less inhibitory than Ag^+.

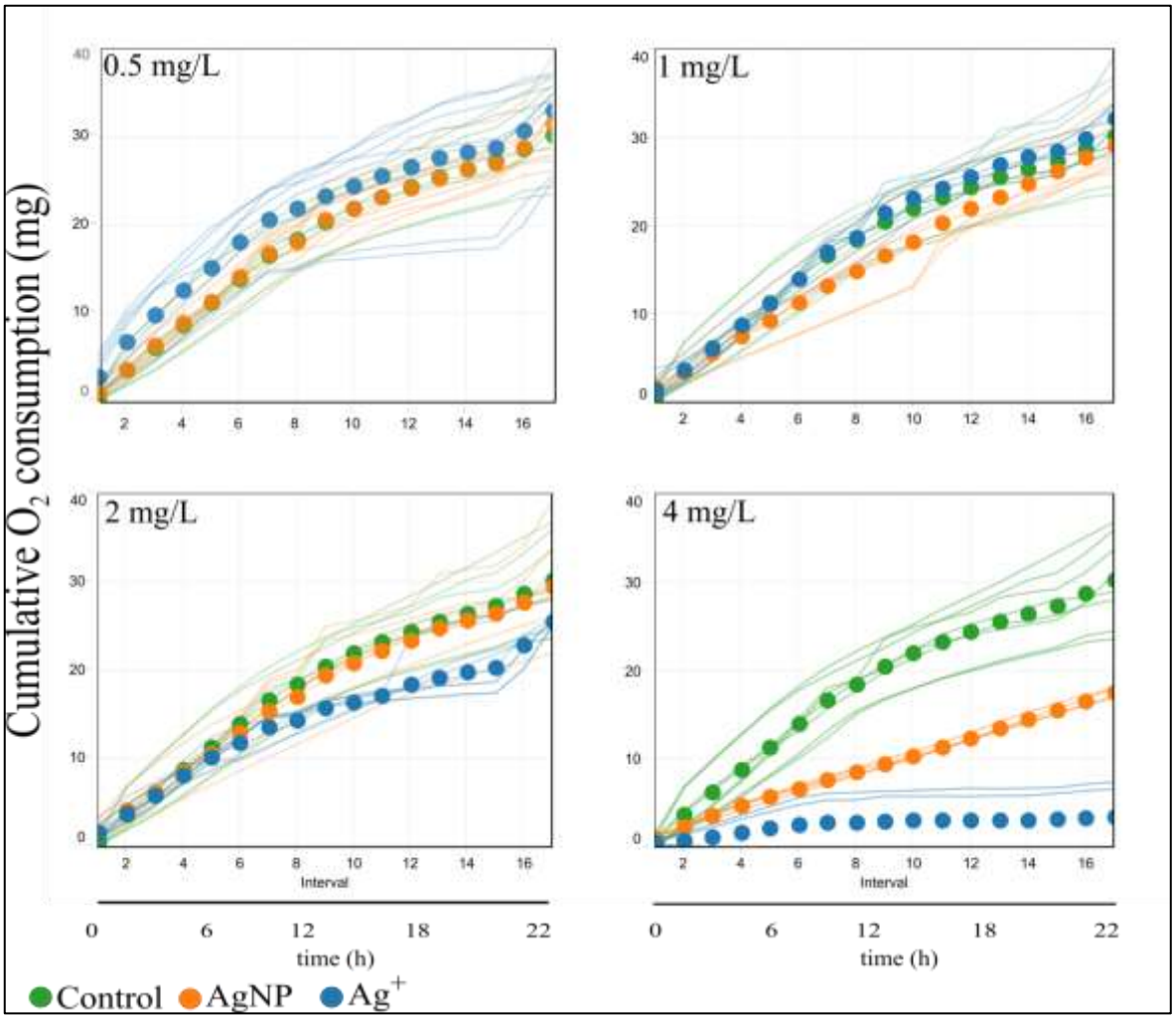


Figure 6.25. Mean cumulative O_2 consumption in the presence of synthesized AgNP and Ag^+ ion (lines represent all measurements, circle markers show the mean values).

IC_{50} values alone do not have an explanatory power of inhibition unless speciation is also considered. In order to have a better insight into inhibition mechanism, Ag^+ speciation was theoretically calculated in terms of free metal, inorganic metal complexes and metal bound to biomass (AgB) using a chemical equilibrium program MINTEQA2 for Windows (Çeçen et al, 2015a,b). Theoretical speciation of Ag^+ in the test medium was calculated. The results are presented in Figure 6.26. As seen in this figure, most of silver was bound to biomass (AgB). Most probably, it was the free form of Ag that was directly taken onto/into biomass causing inhibition, the key species emerge therefore as the free and biomass-bound Ag. The same result was also observed in a former nitrification system (Çeçen et al., 2010a).

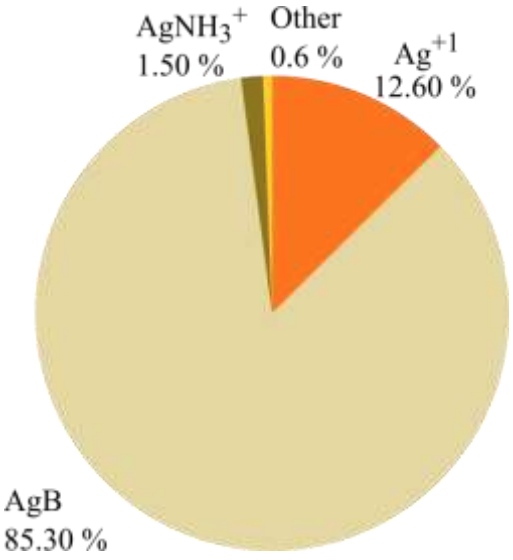


Figure 6.26. Speciation calculations for Ag^{+} with MINTEQA2 in Control (CR) Reactor (Çeçen et al., 2015a).

As observed from experiments, the inhibitory effects of two AgNP solutions (commercial and synthesized) were very different from each other. This discrepancy may arise due to the shape of AgNP as shown in ESEM images in Figure 6.22. The synthesized AgNP was spherical whereas the commercial AgNP had a square-like structure. Studies focusing on the toxicity of AgNP in fish cells indicated that toxicity depended on particle shape (Lee et al., 2007). For example, nanoplates were the most toxic form, nanowires were much less toxic, while nanopsheres were only toxic at high concentrations. In our case, the synthesized AgNP with a spherical shape exerted a medium level of inhibition at 4 mg/L.

Moreover, the zeta potential of nanoparticles is also an important factor affecting toxicity (Silva et al., 2014). Since biomass surfaces are usually negatively charged, in the case of a positive zeta potential, a higher attraction occurs between AgNP and biomass surface. Contrary to this, a higher repulsion occurs when the zeta potential is more negative. In our case, the zeta potential of synthesized AgNP was -25.55±0.88 mV. Within the range of -25 and + 25 mV, a solution is considered stable. Therefore, also this solution was regarded as stable although its negativity was not very high. This characteristic led probably to a medium inhibition as shown in Figure 6.25. A relatively lower attraction is believed to occur between this nanoparticle and biomass. The surface charge of the sludge

varied usually from -0.06 to -0.1 meqv/g MLSS. Considering that the surface charge was related to the ionizable groups on sludge surface, this parameter might be important in the interaction of metals and biomass.

Another important parameter affecting the toxicity was the particle size of AgNP. The particle size of synthesized AgNP was smaller than the commercial one. These particles could probably enter bacterial cells, leading to inhibition. Therefore, oxygen uptakes rates and cumulative O_2 consumption were affected negatively. Different data are reported about the importance of particle size. For example, according to Choi (2008) in the presence of 1 mg/L of AgNP having 14±6 nm average size, 80% inhibition was observed on nitrifiers.

Obviously, in our case the inhibitory effects of Ag^+ and AgNP were not comparable to each other. One of the reasons might be that a very low amount of Ag^+ was released from AgNP (Benn and Westerhoff, 2008) which was believed to be the main species causing toxicity, as also concluded from our former studies (Çeçen et al., 2010a). Further, the inhibitory effect of silver might be reduced if these Ag^+ ions were complexed with ligands such as peptone, acetate or glucose in the feed, or if AgNP itself formed agglomerates (Limbach et al., 2005) or remained in the form of nanoparticles without dissociation (Blaser et al., 2008).

6.3.2.1. Changes in EPS Composition of Activated Sludge in the Presence of Ag^+. In this section, in a respirometric test an activated sludge sample was exposed to Ag^+ ion for about 21 hours and the changes in EPS production were examined. At the end of the test, EPS were extracted from both the control and sample containing Ag^+. Figure 6.27 represents the typical cumulative oxygen consumption in such tests.

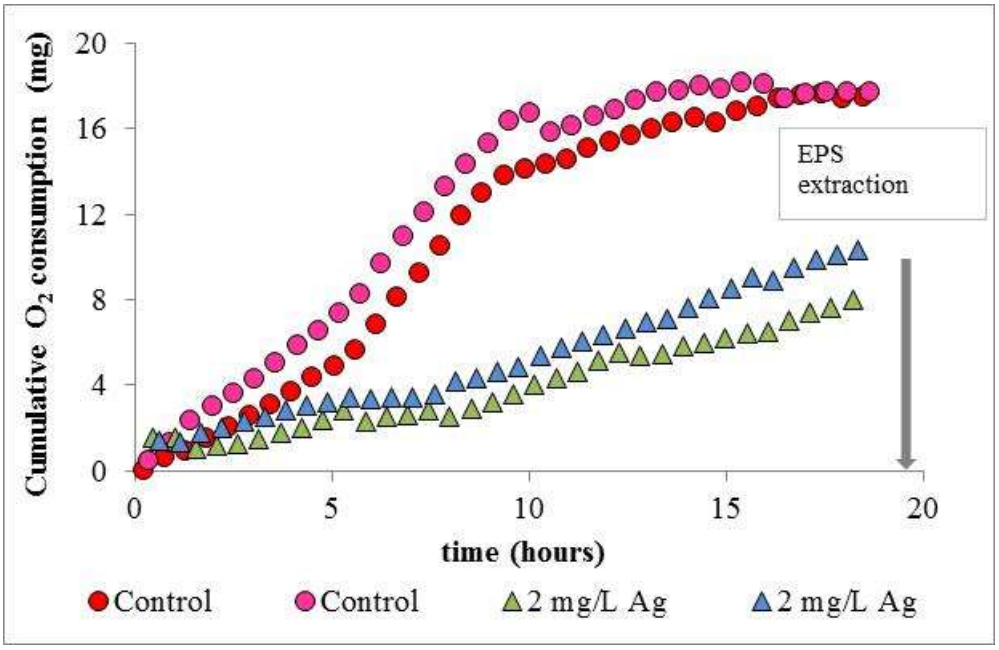


Figure 6.27. Cumulative O_2 consumption of activated sludges in the presence and absence of Ag^+.

EPS were measured by colorimetric methods and results were presented in Table 6.5. According to results, TB-EPS was generally decreased in the presence of Ag^+ ion in activated sludge samples. On the other hand, even a small increase was observed in VLB-EPS and LB-EPS fractions. After the exposure to Ag^+, microorganisms produced more looser EPS fractions, probably to diminish the inhibitory effect of silver.

In the presence of Ag^+, deterioration of the protein in EPS structure was likely to occur. Therefore, the proteins in different EPS fractions were examined by HPSEC. As an example, chromatograms belonging to the VLB-EPS fraction are presented in Figure 6.28. As seen in this figure, 2 mg/L Ag^+ had a negative effect on the proteins in VLB-EPS. Chromatograms were splitted and the absorbance peaks (mAU value) decreased. All this indicated the structural deterioration of protein-EPS. This deterioration was probably observed due to the complexation of Ag^+ ions with the functional groups on proteins.

Table 6.5. Effect of Ag^{+} addition in short-term respirometric tests on production of EPS.

Set #	Sample	Carbohydrate (mg/g MLVSS)				Protein (mg/g MLVSS)				Total EPS (mg/g)
		VLB-EPS	LB-EPS	TB-EPS	Total EPS	VLB-EPS	LB-EPS	TB-EPS	Total EPS	
1	Control	0.291	0.158	1.664	2.113	0.250	0.232	4.760	5.242	7.355
	2mg/L Ag	0.000	0.106	1.100	1.206	0.113	0.587	2.390	3.090	4.296
2	Control	0.321	0.254	3.530	4.105	1.733	0.684	4.330	6.747	10.852
	2mg/L Ag	0.417	0.328	5.722	6.467	0.765	0.891	3.620	5.276	11.743
3	Sludge	0.580	0.447	1.546	2.573	0.465	0.573	1.782	2.820	5.393
	Control	0.411	0.306	1.174	1.891	0.528	0.313	1.768	2.609	4.500
	2mg/L Ag	0.743	0.343	0.804	1.890	0.402	0.521	0.878	1.801	3.691
4	Control	0.315	0.346	1.360	2.021	0.608	0.513	1.836	2.957	4.978
	2mg/L Ag	0.675	0.453	1.004	2.132	0.425	0.421	1.242	2.088	4.220
5	Control	0.384	0.357	0.996	1.737	0.615	0.613	1.968	3.196	4.933
	2mg/L Ag	0.678	0.454	0.798	1.930	0.502	0.580	1.358	2.440	4.370
6	Control	0.500	0.313	1.300	2.113	0.518	0.413	1.517	2.448	4.561
	2mg/L Ag	0.643	0.643	0.804	2.090	0.682	0.629	1.028	2.339	4.429

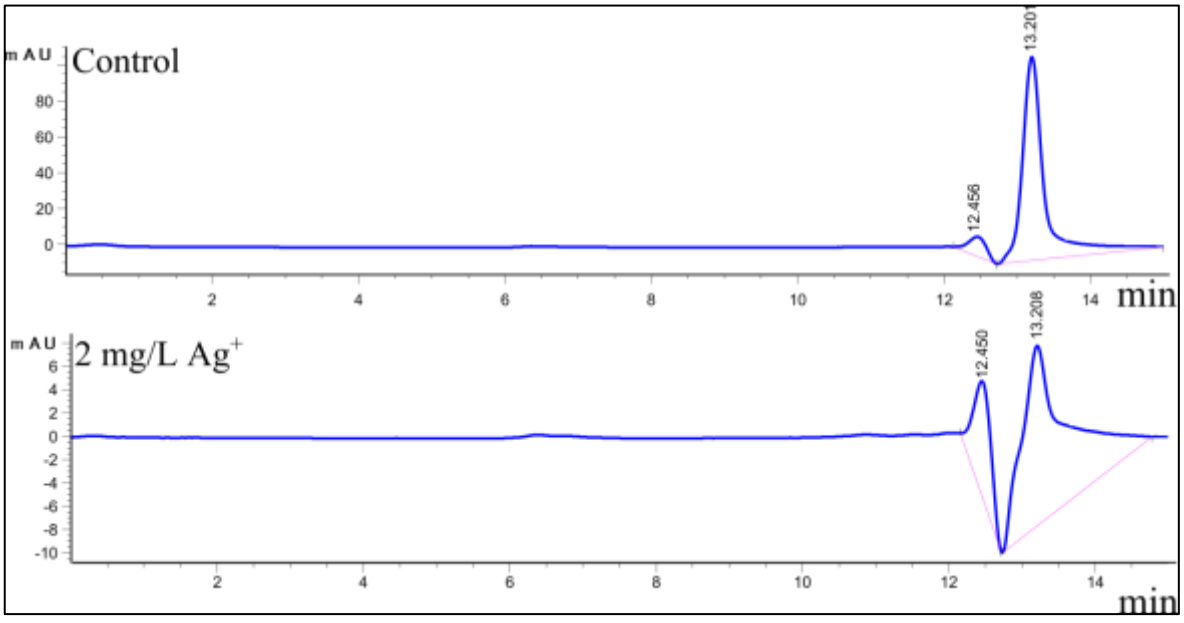


Figure 6.28. HPSEC chromatograms belonging to VLB-EPS fractions in control and Ag^{+} added samples.

6.3.2.2. Complexation of Ag^+ and AgNP with Different EPS Fractions. Binding of AgNP and Ag^+ to the different fractions in EPS were determined. For this purpose, three different EPS fractions formerly uncontacted with silver species (6.3.1), were exposed to either 2 mg/L AgNP or Ag^+. Figure 6.29a represents a typical EPS-AgNP voltammogram. The complexation of EPS fractions with silver species was measured by voltammetry.

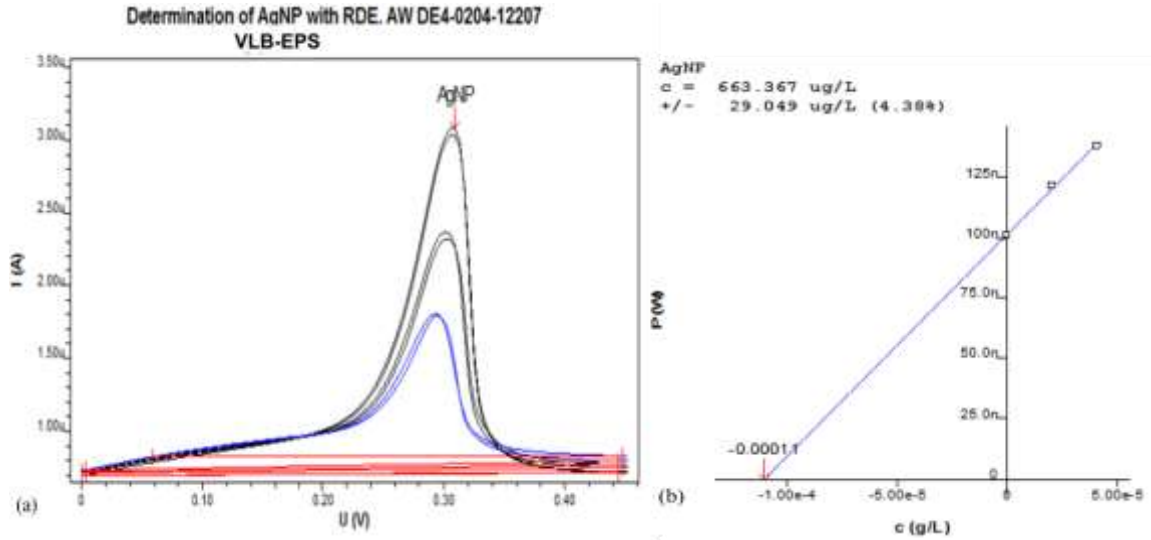


Figure 6.29. Voltammetric analysis:
a) Typical voltammogram in the case of contact of EPS with AgNP,
b) Calculation of AgNP concentration by standard addition.

After short-term contact (15 min) of EPS with silver, the remaining silver was measured by VA. Thus, it was possible to measure the silver remaining in the form of free ion and weak complexes. The difference between the total silver and the silver in solution showed the amount of silver that was complexed with the EPS fraction. Figure 6.30 illustrates the complexed metal in each EPS fraction.

According to results, the complexation of Ag^+ and AgNP was higher with the VLB-EPS fraction. The affinity of Ag^+ and AgNP to VLB-EPS was almost the same. On the other hand, this affinity was different in LB-EPS ve TB-EPS fractions. Results demonstrated that LB-EPS and TB-EPS fractions had a tendency to complex more with AgNP than Ag^+.

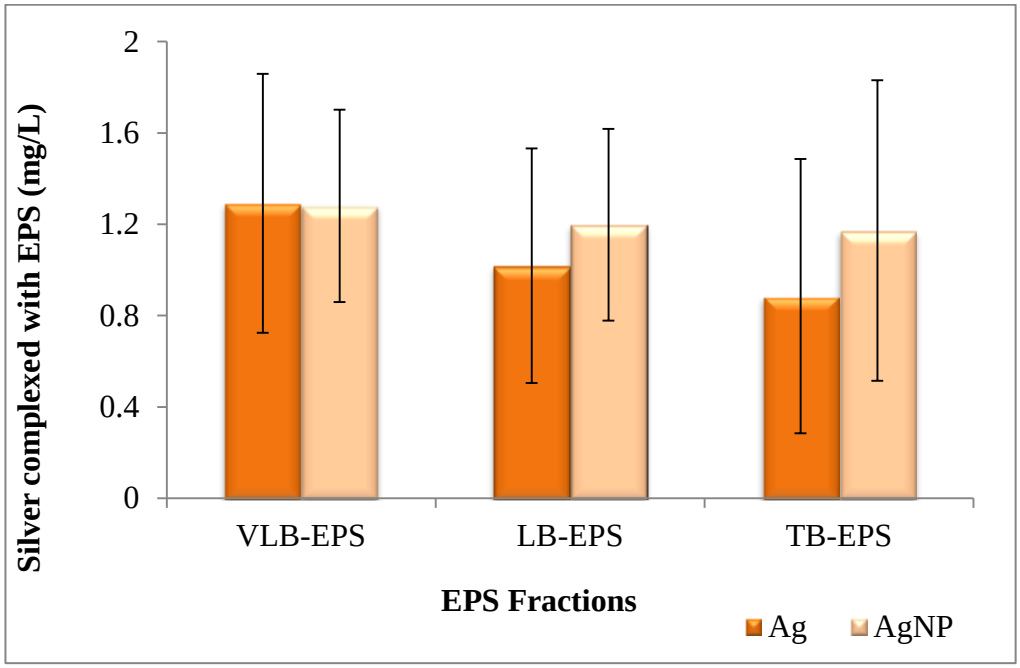


Figure 6.30. Complexation of Ag^+ and AgNP with different EPS fractions (initial Ag conc: 2 mg/L) (n=5) (Geyik and Çeçen, 2014b).

One of the important factors determining the complexation of Ag^+ and AgNP was the MW of proteins in EPS. In all fractions, EPS having relatively small proteins (7-16 kDa) possessed the maximum EPS-Ag^+ complexation capacity. On the other hand, when very small proteins were dominant (3-8 kDa), then EPS complexed more with AgNP. In general, AgNP tended to bind EPS with smallest MW.

6.3.3. Long-Term Exposure of Different Activated Sludges to AgNP

Based on a previous work of our group (Çeçen and Kılıç, 2015), the inhibitory effect of Ag^+on CR, RG and RP sludges was different as calculated from respirometric experiments. Results demonstrated that the sludges CR and RG were similarly inhibited in the presence of Ag^+. On the other hand, RP sludge proved to be much more resistant to Ag^+ due to complexation of Ag^+ with the compounds in peptone. These sludges also had different surface properties as indicated in Section 6.2.2.

Under the light of these findings, long-term experiments were designed to show the inhibitory effect of AgNP. RP and CR sludges were selected since they had different EPS

and surface properties. It was expected that these sludges responded differently under long-term exposure to AgNP.

For this purpose, two new reactors were started up with the sludge taking from CR and RP reactors. These reactors received also AgNP and were named as CR_{AgNP} and $RP_{AgNP.}$ They were operated in parallel to CR and RP reactors containing no AgNP. When these long-term experiments were started, CR and RP sludges were already in steady-state conditions. The main aim here was to monitor the effect of AgNP on activated sludge systems receiving different type of synthetic wastewaters.

6.3.3.1. Effect of AgNP on Peptone Reactors. In this part, the reactor operation was divided into four phases:

Phase I: This phase covered 0-78 days of operation. RP and RP_{AgNP} were compared in terms of COD removal, EPS production, MLVSS and surface properties. In this phase, both reactors were run in parallel without any AgNP addition.

Phase II: In this phase, RP_{AgNP} reactor was semi-continuously supplied with AgNP with Feed P. The reactor having 2 L of active volume was fed every second day. In each semi-continuous run, 0.3 mg AgNP was added to the reactor. This phase covered between 79 and 155 days of operation. At the end, total AgNP concentration in the reactor was raised from 0 mg/L to 0.8 mg/L.

Phase III: In this phase covering 156-238 days of operation, AgNP dose was increased to 2 mg. At the end of this phase, total AgNP concentration in the reactor was raised from 0.8 mg/L to 12.4 mg/L.

Phase IV: In the last phase, AgNP concentration was further increased to 10 mg, and at the end of the phase, AgNP in the mixed liquor RP_{AgNP} was 33.7 mg/L as shown in Figure 6.31.

As seen in Figure 6.31, in Phases III and IV, AgNP concentration was raised to much higher concentrations than found in domestic wastewater. Even at such concentrations, 90

% of COD was successfully degraded. When RP_{AgNP} sludge was exposed to AgNP first, MLVSS concentration decreased sharply as seen in Figure 6.32. Almost 50 % of biomass was lost and MLVSS did not increase again at the end of Phase III. At the end of Phase II, RP_{AgNP} sludge had first a greyish color, then it became dark grey (Figure 6.33). Most probably, AgNP lost its stability due to agglomeration of particles.

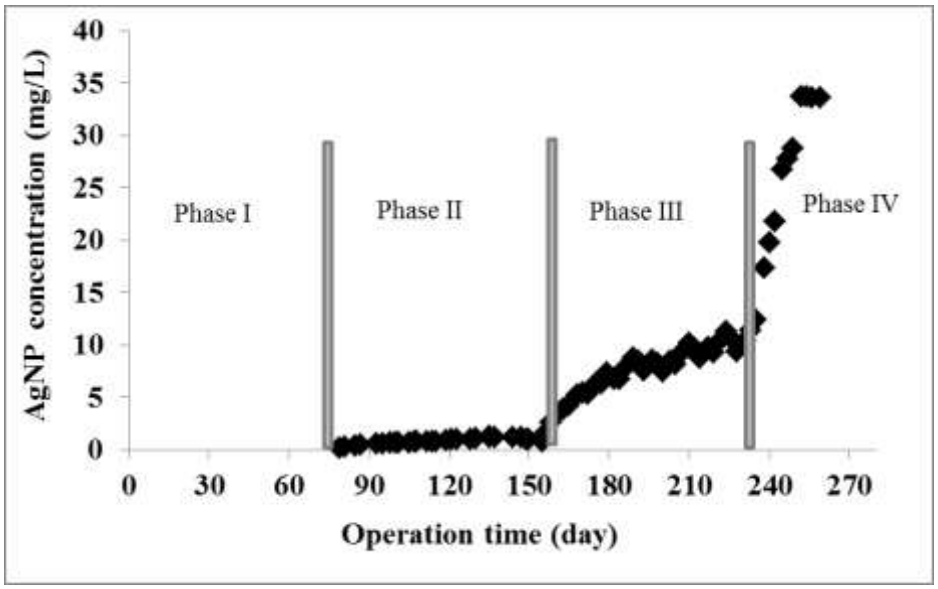


Figure 6.31. AgNP concentration in reactor RP_{AgNP}.

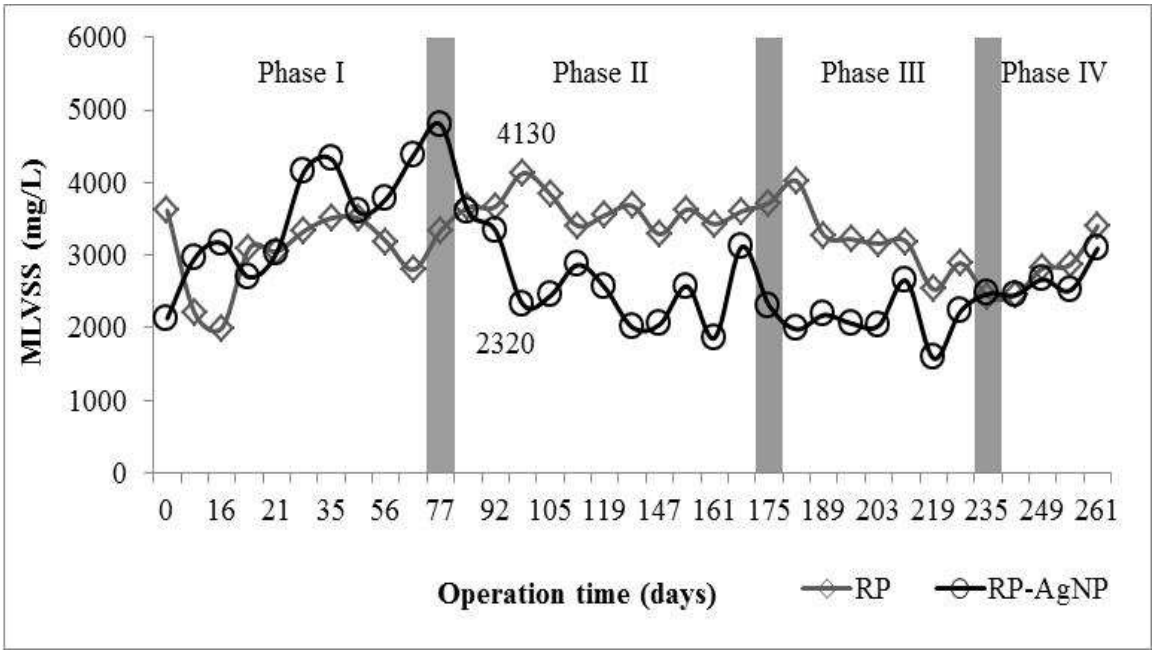


Figure 6.32. Changes in MLVSS concentration in RP and RP_{AgNP} reactors.



Figure 6.33. Images of RP and RP_{AgNP} sludges, left: Pepton Reactor (RP), right: Pepton Reactor (RP_{AgNP}) receiving also AgNP.

According to results, continuous AgNP addition did not affect oxygen uptake rate (OUR) of microorganisms. These results are consistent with literature (Zhang et al., 2014). Figure 6.34 shows two respirograms belonging to days 156 and 226 in Phase III. At 156 days of operation, total AgNP concentration in the reactor was about 1.1 mg/L and OUR values showed that organic carbon removal and nitrification were not affected.

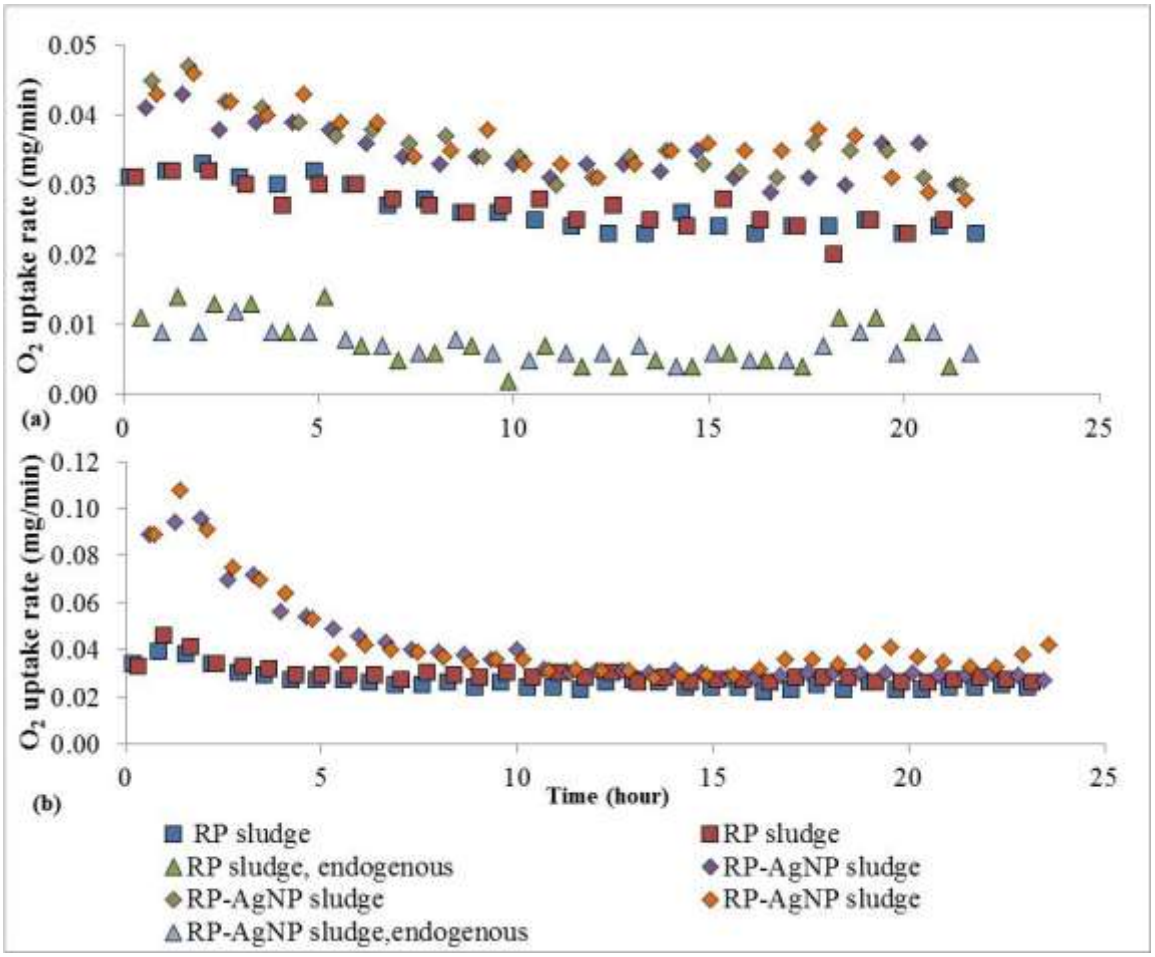
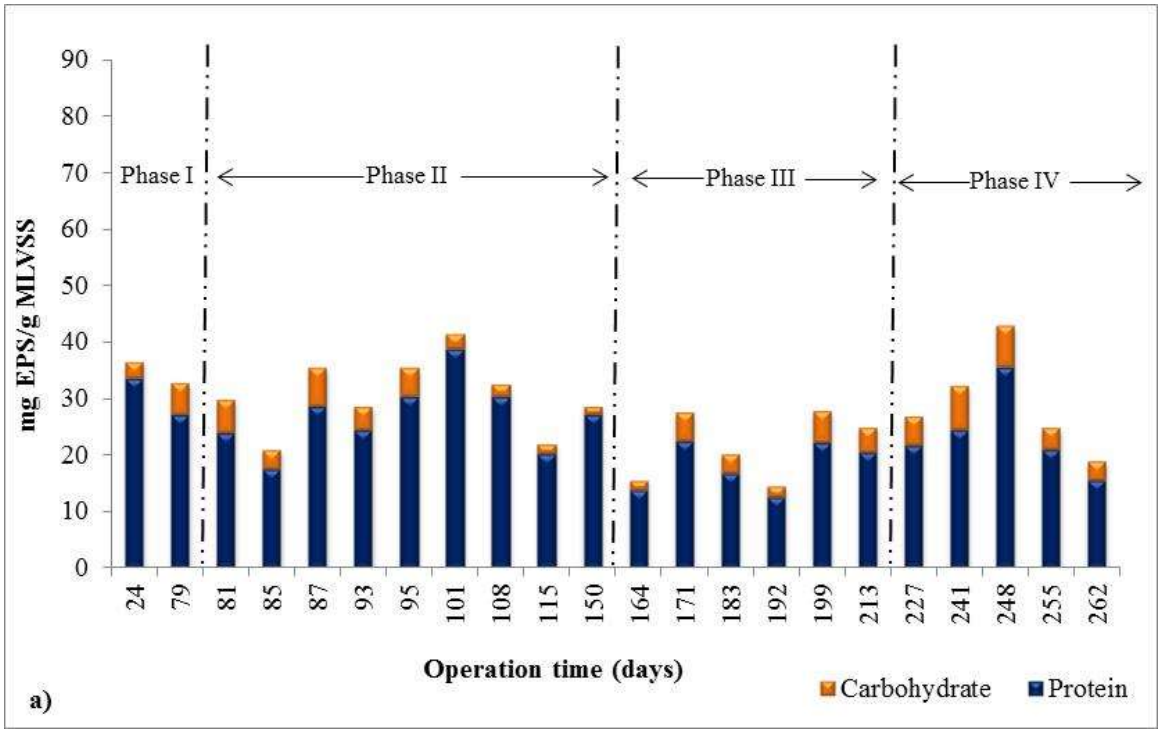


Figure 6.34. Oxygen uptake rates (OUR) of RP and RP_{AgNP} sludges at a) Day 156 and b) Day 226.

With time, AgNP accumulated in the reactor and reached 5.3 mg/L at 226 days. At the start of the experiment, readily biodegradable COD arising from peptone was essentially consumed in about 2 hours, then slight decrease was observed in OUR in both reactors. Then, 10 hours later all of the organic matter was consumed in reactors. In other words, 5.3 mg/L AgNP did not affect peptone degradation.

In fact, if the sludge were inhibited, there would be a reduction in oxygen consumption. However, in this study there was not such a reduction. The substrate (peptone) was utilized and AgNP had no acute or chronic effect on biodegradation of peptone in the RP_{AgNP} reactor.

6.3.3.2. Effect of AgNP on Production of EPS in the RP_{AgNP} Reactor. Long-term addition of AgNP did not affect composition of EPS as seen in Figure 6.35. Both in RP and RP_{AgNP} sludges proteinaceous-EPS was dominant while carbohydrate-EPS production was lower than 5 mg/g MLVSS until Phase IV. However, the EPS yield was highly affected when AgNP concentration was increased in the reactor. EPS of RP_{AgNP} reactor exhibited higher fluctuations upon continuous exposure to AgNP. In particular, there were high fluctuations in proteinaceous-EPS (Figure 6.35). Both carbohydrate- and protein-EPS were affected as a result of exposure to higher AgNP concentrations. Up to Phase III, Protein-EPS were about 23.4±6.8 mg/g MLVSS and 26.9±9.6 mg/g MLVSS in RP and RP_{AgNP}, respectively. In RP_{AgNP} sludge, EPS were only 14 % higher than in the RP sludge. But, total-EPS production became higher at increased AgNP concentrations as seen in Phases III and IV (Figure 6.35). In the last phase (Phase IV), in RP_{AgNP} sludge, EPS production was twice higher than in RP.



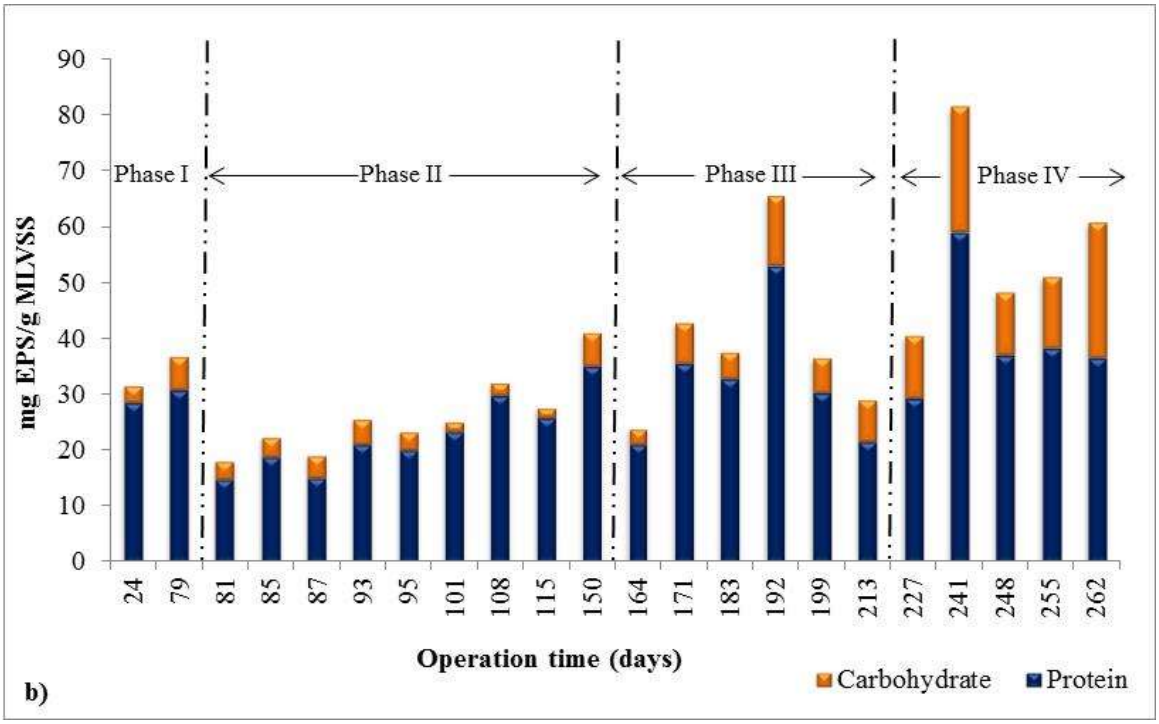


Figure 6.35. Carbohydrate and proteins in the EPS of peptone reactors a) Reactor RP, b) Reactor RP_{AgNP} (with AgNP addition)

RP_{AgNP} reactor was operated for 79 days without AgNP addition. As seen from Figure 6.36, all fractions, namely VLB-EPS, LB-EPS and TB-EPS were produced approximately at the same level as in reactors run in parallel (RP and RP_{AgNP}).

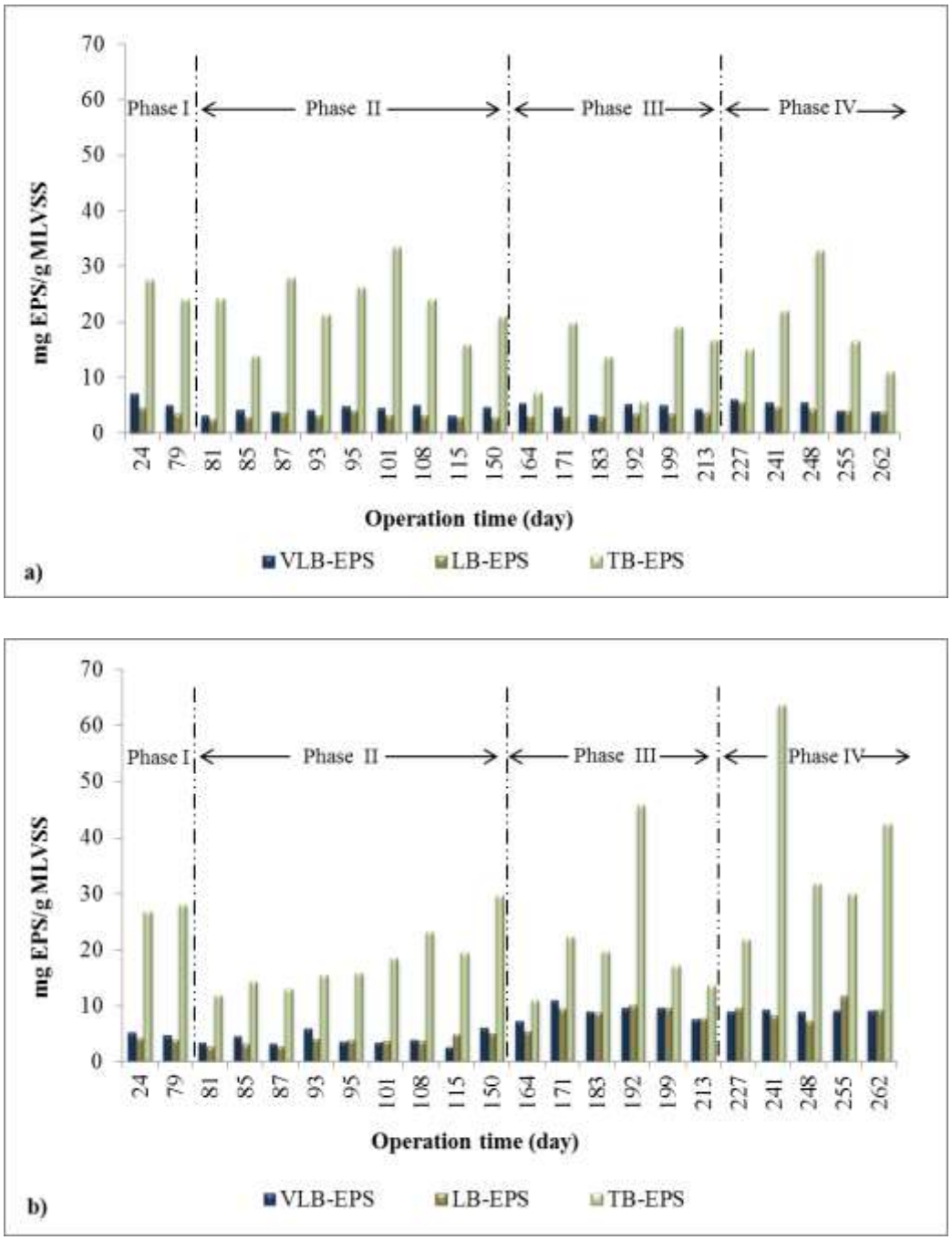


Figure 6.36. Comparison of EPS fractionation in peptone reactors a) RP and b) $RP_{AgNP.}$ (VLB-EPS:Very Loosely Bound-EPS, LB-EPS: Loosely Bound-EPS, TB-EPS: Tightly Bound-EPS).

When biomass was first exposed to AgNP, at the beginning of Phase II, EPS production was sharply decreased, in particular in TB-EPS fraction. Then, TB-EPS gradually increased as shown in Figure 6.36, when total AgNP reached 0.7-0.8 mg/L. However, still no change was observed in EPS fractionation. The fractionation of EPS began to change at the end of Phase II. The production of LB-EPS in RP_{AgNP} slightly increased compared to RP sludge as shown in Figure 6.36.

Results showed that (i) 0.8 mg/L AgNP did not affect EPS production (ii) 0.8 mg/L AgNP had no inhibitory effect on carbon and nitrogen removal since the nano Ag^{+} ion concentration was probably much lower than AgNP concentration. The overall conclusion was that (a) lower concentrations of AgNP could be tolerated by biomass by changing the EPS production and (b) fractionation of EPS did not change much at such AgNP concentrations.

In Phase III, still no changes were observed in terms of organic carbon removal. However, a clear change was recorded in EPS. In this phase AgNP concentration was in the range of 0.8-12.4 mg/L. When microorganisms were exposed to higher levels of AgNP, their EPS production initially dropped as a response. This happened both in Phases II and III at the first high dose. Then, in each phase a gradual increase was observed in EPS production. In Phase III, the production of VLB-EPS and LB-EPS production increased. There was also an increase in TB-EPS as shown in Figure 6.37. However, higher fluctuations were observed in this fraction. Total-EPS production was twice higher than in RP sludge. It is well documented that in the presence of toxic substances soluble EPS production increases in aerobic or in anaerobic systems (Aquino and Stuckey, 2004). Also, in Phase III of the present study looser EPS fractions were produced in higher amounts.

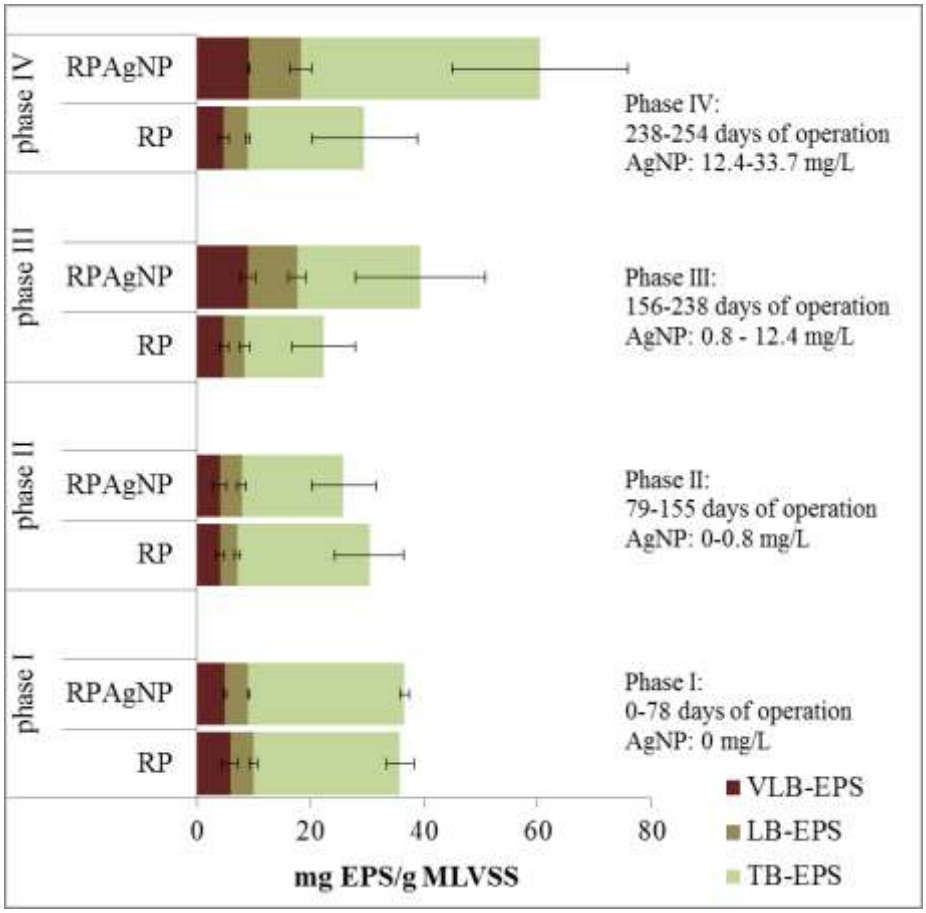


Figure 6.37. Fractionation of EPS samples in different phases of RP and RP_{AgNP} operation (n=22) (VLB-EPS: Very Loosely Bound-EPS, LB-EPS: Loosely Bound-EPS, TB-EPS: Tightly Bound-EPS).

Further increase in AgNP concentration in Phase IV (Figure 6.37) led to a higher TB-EPS production. Possibly, biomass increased its TB-EPS production to diminish the inhibitory effect of AgNP. In this phase, MLVSS concentration in RP_{AgNP} reached approximately the same value as in RP. Moreover, with time AgNP lost its stability and was converted into metallic form. Therefore, it did not exert any toxic effect on sludge.

Overall, EPS production did not change up to 5 mg/L of AgNP. However, such high levels of AgNP are not possible in a real wastewater treatment system. The main result of the study is that EPS were protecting microorganisms from toxic effects of AgNP.

Previous research indicated that about 90 % of spiked AgNP were efficiently removed by biological treatment under controlled laboratory conditions (Shafer et al., 1998; Kaegi et al., 2011). Li et al., (2013) showed that, AgNP can be removed by mechanical treatment, the remaining part was removed biologically up to 72.3−99.3 %. Studies showed that approximately 95% of AgNP entering municipal WWTPs was removed from wastewater. On the other hand, AgNPs were likely to accumulate on biosolids that are usually used as agricultural land amendments, placed in landfills or incinerated. The biosolids may present a potential source for AgNP release into the environment that is very different from WWTP liquid discharge (Liu et al., 2010; Glover et al., 2011; Maurer et al., 2012).

6.3.3.3. Effect of Continuous AgNP Addition on the Surface Properties of Sludge. Cell surface hydrophobicity has been considered as a significant factor for the stability of microbial aggregates (Liu et al., 2004). In literature, it is indicated that EPS could decrease the negative charge of cell surfaces, therefore two neighbouring cells physically bridge to each other (Shen et al., 1993). Using a colloidal titration technique, in this study activated sludge was found to be negatively charged as seen in Figure 6.38. Considering that the surface charge is related to the ionizable groups on sludge surface, this parameter may become important in interaction of metals with biomass.

In Phase II, the surface charge of sludge significantly increased with accumulation of AgNP in RP_{AgNP} as indicated in Figure 6.38. In Phase III, an increase was observed in TB-EPS production both in protein-EPS and carbohydrate-EPS content. At the same time, hydrophobicity of sludge decreased and surface charge became less negative. Then, hydrophobicity stayed at lower levels but surface charge became more negative when AgNP accumulated at about 4.3 mg/L in the reactor. At the same time, EPS production, in particular the production of VLB-EPS and LB-EPS fractions became higher as a response to this inhibitory substance. It is thought that higher VLB-EPS and LB-EPS production supported the integrity of bacteria in the presence of AgNP.

AgNP itself has a certain surface charge and hydrophobicity which were about -0.309 meqv/g AgNP and 25 %, respectively. With accumulation of AgNP having higher

surface charges and lower hydrophobicities than the sludge itself, sludge surface characteristics would be affected.

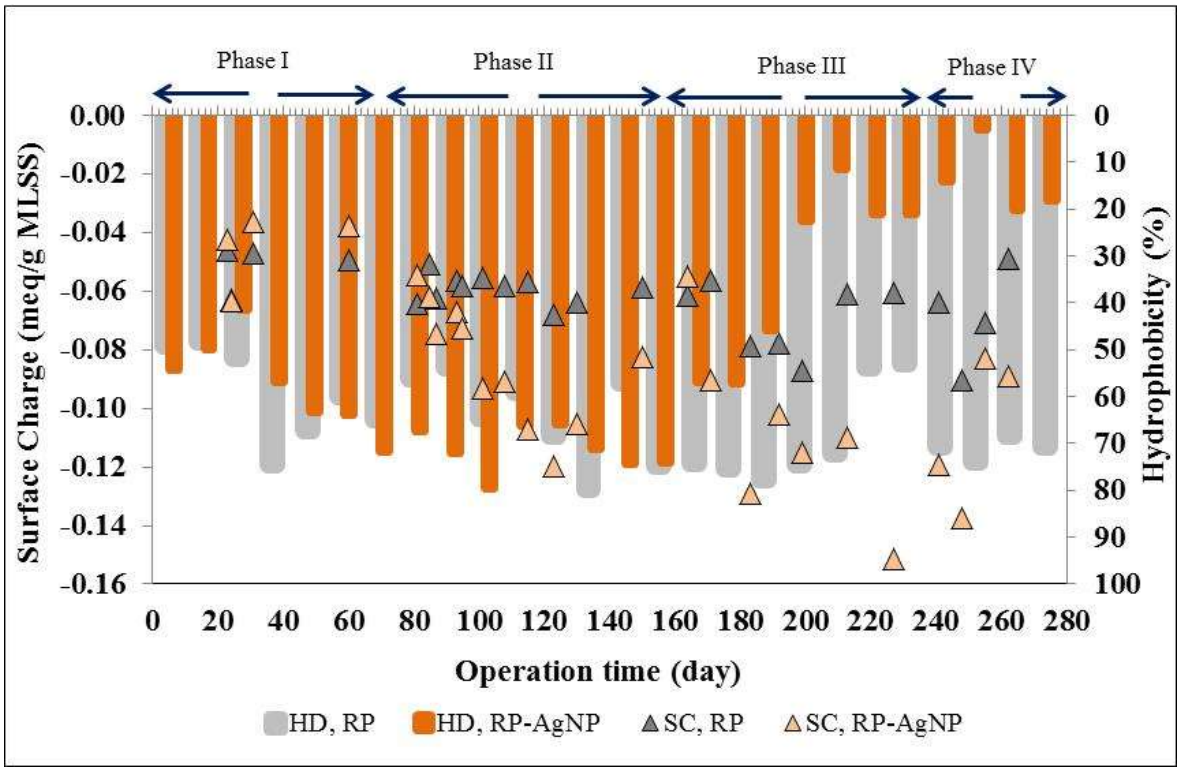


Figure 6.38. Cell surface charge and hydrophobicity of sludges fed with peptone. RP: Peptone Reactor, RP_{AgNP}: Peptone Reactor receiving AgNP. SC: Surface Charge, HD: Hydrophobicity.

Due to addition of AgNP which has a lower hydrophobicity and a more negative surface charge than the RP sludge itself, the RP_{AgNP} became less hydrophobic and more negative at the end. Results showed that surface charge and hydrophobicity were inversely correlated to each other in the Peptone reactor (RP) (Section 6.2.2). The surface charge is related to the presence of ionizable groups on sludge surface; the presence of these groups increases the polar interactions of EPS with water molecules. Therefore, the more charged the sludge surfaces are, the lower is the hydrophobicity (Liao et al., 2001).

6.3.3.4. Changes in the Molecular Weight of Protein-EPS. During operation of RP and RP_{AgNP}, EPS were analyzed by HPSEC. The data presented in Figure 6.39 shows the MW of proteins and reflect the results of about 290 measurements.

In Phase I, RP_{AgNP} did not receive AgNP. Protein-EPS in this sludge had small MWs lower than 13.6 kDa. Proteins with higher MWs were observed in TB-EPS fractions only.

In Phase II, proteins in Sp-EPS and VLB-EPS fractions had the same MW, both in RP and RP_{AgNP}. In RP sludge, only one type of protein was produced in the LB-EPS fraction. On the other hand, three types of proteins existed in RP_{AgNP} sludge. It is thought that new proteins were produced in the MW ranges of 1571-2241 and 28-48 kDa in the presence of AgNP. In this phase, AgNP concentration was in the range of 0.15-0.8 mg/L. RP_{AgNP} sludge could tolerate 0.8 mg/L of AgNP by producing new proteins in LB-EPS. According to colorimetric results shown in Figure 6.35, there was not an obvious difference between RP and RP_{AgNP} sludges in terms of EPS production in Phase II.

In Phase III, the percentage of the smaller proteins in Sp-EPS increased in RP_{AgNP} sludge. In LB-EPS, the diversity of proteins increased to four in RP_{AgNP} sludge. It was believed that these newly emerging proteins played an important role in complexation. The proteins in TB-EPS fractions were identical in both sludges. HPSEC fingerprints and colorimetric results showed that with increasing AgNP concentration, first VLB-EPS (Phase III) and LB-EPS (Phase III) production increased.

In Phase IV, Sp-EPS having MW of 8.8 kDa were dominant in both sludges. In RP, VLB-EPS and LB-EPS, the MWs of proteins were less than 8.8 kDa, while in RP_{AgNP} slightly larger proteins were detected. These higher proteins might be seen as a result of cell lysis at increased AgNP concentration. The greatest difference between the sludges was seen in TB-EPS. Two different types of proteins were detected in RP sludge. On the other hand, five types of proteins were observed in RP_{AgNP} sludge. Compared to other phases, in RP_{AgNP} sludge TB-EPS production was approximately twice higher than RP sludge.

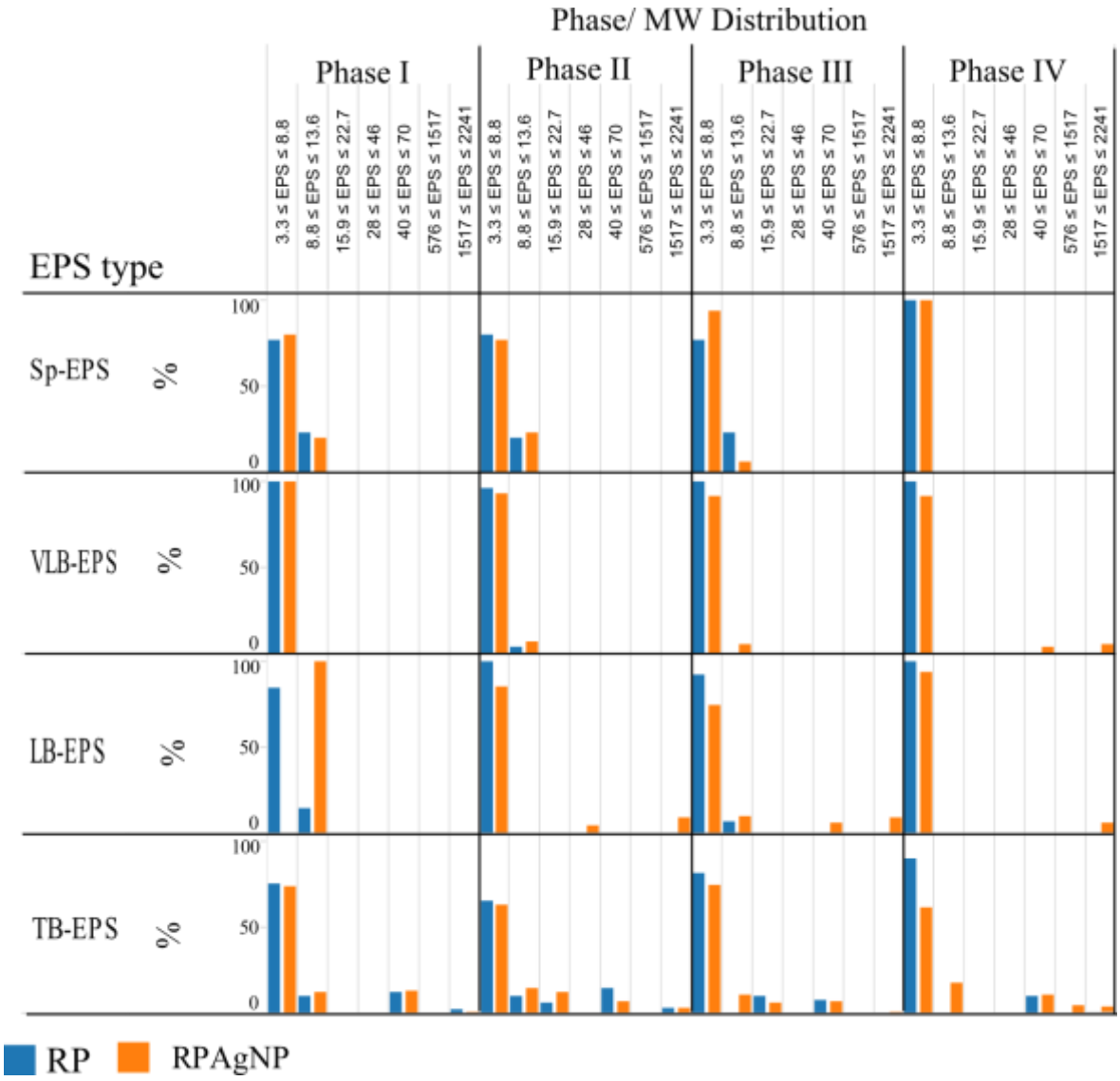


Figure 6.39. MW distribution of EPS in RP and RP_{AgNP} sludges in different phases of reactor operation.

As a result, in the presence of AgNP the first response of microorganisms was an increase production of VLB-EPS and LB-EPS. Both HPSEC measurements and colorimetric results were in agreement with each other. Further increase in AgNP concentration led also to an increase in TB-EPS. Moreover, the type of protein-EPS in each fraction changed as a response to AgNP. Increase in EPS production might enable bacteria to protect themselves from toxic effects of AgNP.

6.3.3.5. Comparison of Control Reactor (CR) and Control Reactor receiving AgNP (CR_{AgNP}).

The reactor operation was divided into four phases:

Phase I: This phase covered 0-34 days of operation. CR and CR_{AgNP} were compared in terms of COD removal, EPS production, MLVSS and surface properties. In this phase, the reactors were run in parallel without any AgNP addition.

Phase II: In this phase, CR_{AgNP} reactor was supplied with AgNP along with Feed 1. The reactor having 2 L of active volume was fed every second day. In each semi-continuous run, 0.3 mg AgNP was added to the reactor. This phase covered between 34 and 107 days of operation. At the end, total AgNP concentration in the reactor was raised from 0 mg/L to 1.0 mg/L.

Phase III: In this phase covering 107-139 days of operation, AgNP dose was increased to 2 mg. At the end of this phase, total AgNP concentration in the reactor was raised from 1.0 mg/L to 12.7 mg/L.

Phase IV: In the last phase, AgNP concentration was further increased to 10 mg. At the end of the phase AgNP concentration inside CR_{AgNP} reached 23.1 mg/L as shown in Figure 6.40.

As seen in Figure 6.40, AgNP was raised again to much higher concentrations than found in domestic wastewater. At such concentrations, 90 % of COD was successfully degraded. Contrary to RP_{AgNP} sludge, when CR_{AgNP} was first exposed to AgNP, MLVSS concentration did not decrease. During the whole operation period CR and CR_{AgNP} had approximately the same MLVSS except in the middle of Phase II as seen in Figure 6.41.

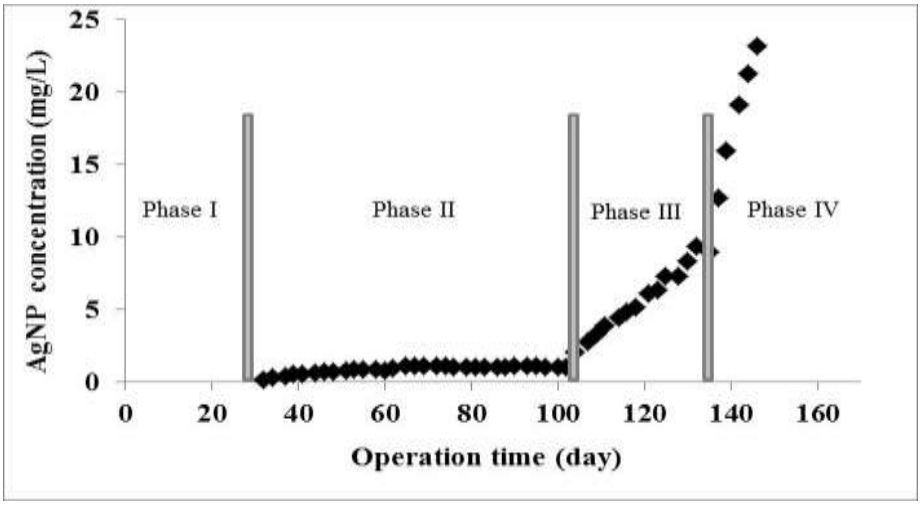


Figure 6.40. AgNP concentration in the Control Reactor receiving AgNP (CR_{AgNP}).

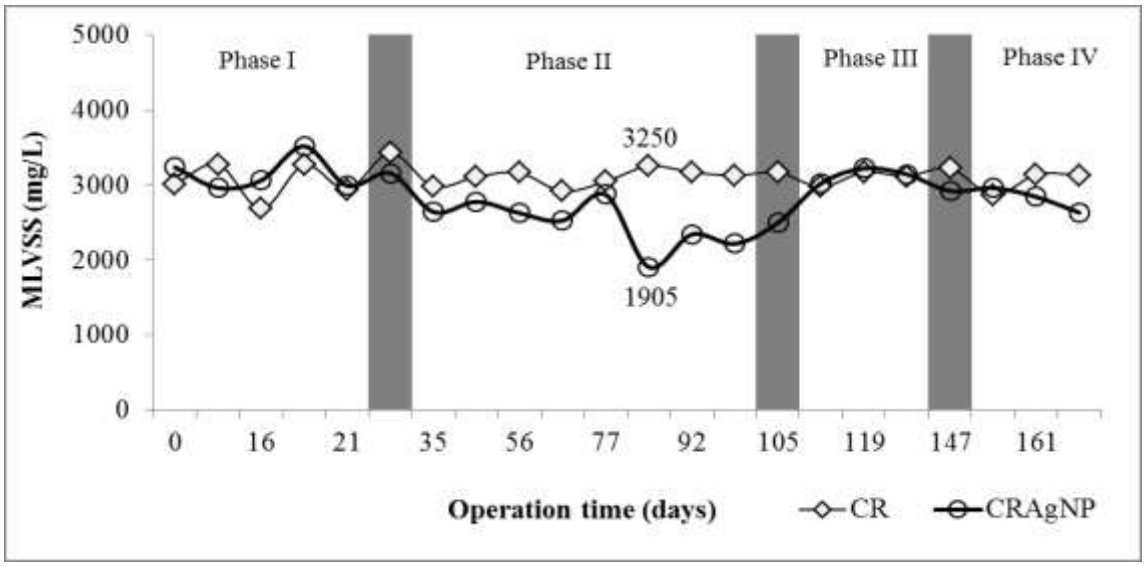


Figure 6.41. Changes in the MLVSS concentration of the reactors CR and CR_{AgNP}.

According to respiration studies, continuous AgNP addition slightly affected oxygen uptake rates (OUR) of biomass in CR_{AgNP}. Figure 6.42 shows two respirograms belonging to days 53 and 149, in Phase II and IV, respectively. At day 53, there was approximately 70 % reduction in OUR, when microorganisms were exposed to 0.8 mg/L of AgNP. As concluded from a previous MSc.Thesis (Kılıç, 2014), it is thought that the nitrifier fraction is influenced by AgNP addition. In such a case, in a real WWTPs, nitrification might be negatively affected upon continuous exposure to AgNP.

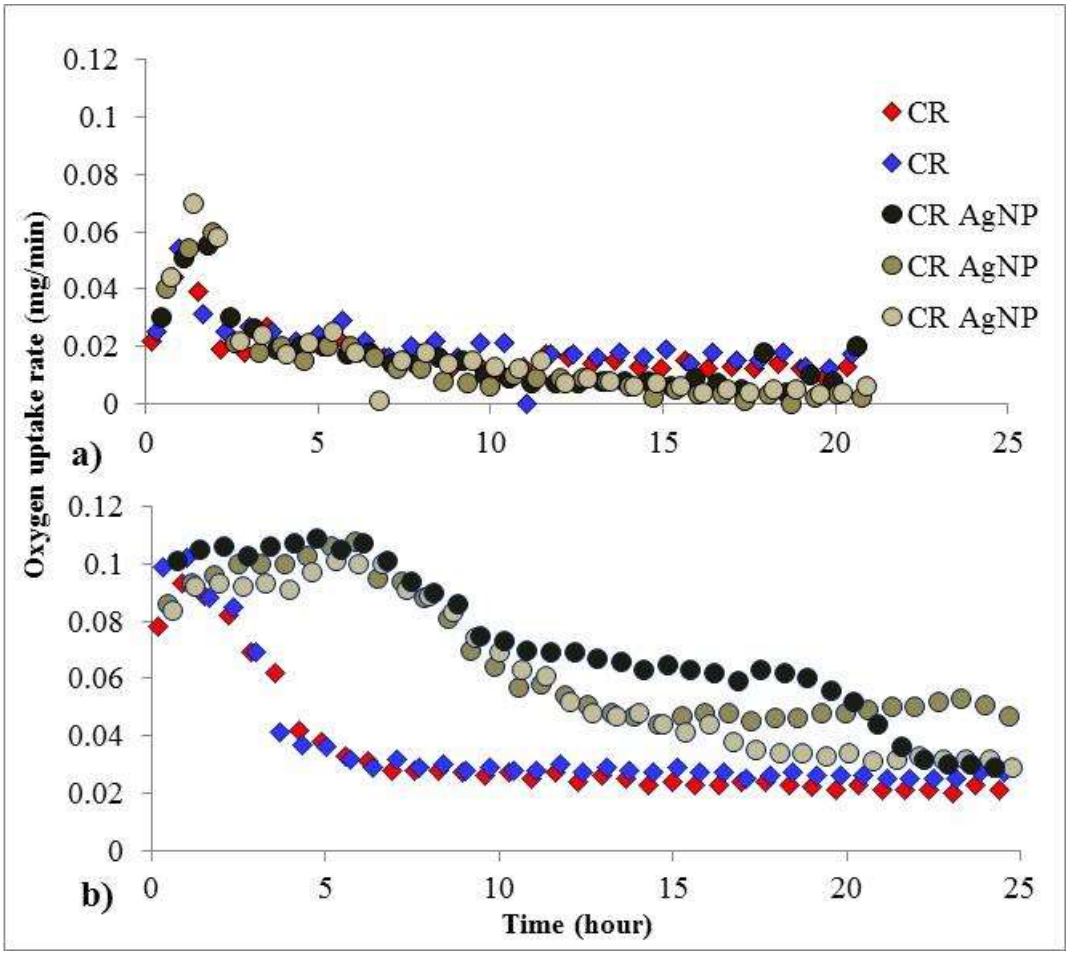


Figure 6.42. Oxygen uptake rates of CR and CR_{AgNP} sludges at a) Day 53 and b) Day 149.

6.3.3.6. Effect of AgNP on EPS Production and Surface Properties of CR_{AgNP} Sludge. Protein-EPS were dominant in CR and CR $_{AgNP}$ sludges. When biomass was first exposed to AgNP in Phase II, EPS production decreased. Then, the production increased fourfold (Figure 6.43). In general, EPS production was generally higher in CR_{AgNP} compared to CR, but EPS production fluctuated in all phases. It is thought that EPS production increased only if AgNP could not be tolerated by biomass. When the accumulated AgNP lost its stability by agglomeration and was converted into a noninhibitory form, also EPS production of biomass did not increase.

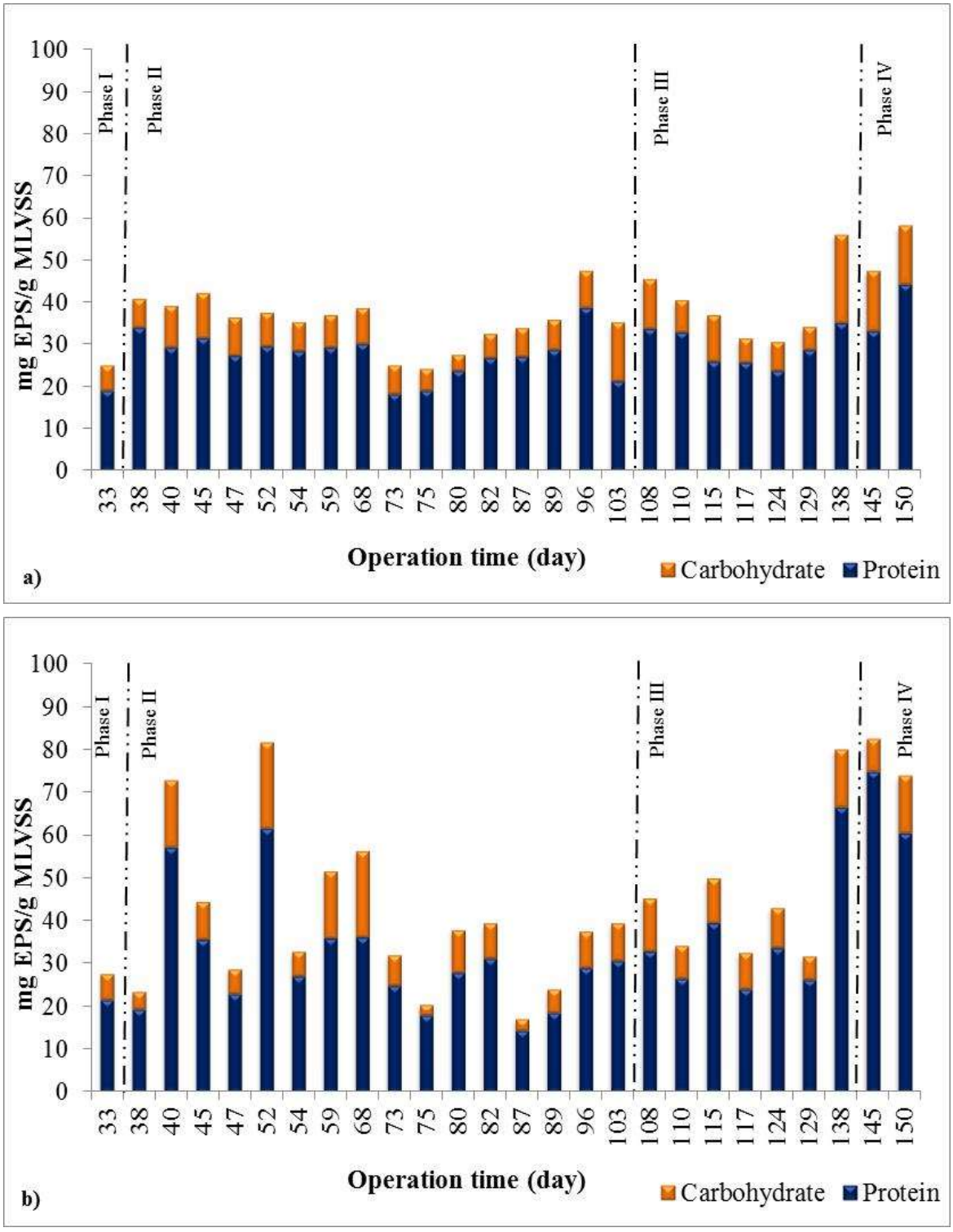


Figure 6.43. EPS production in a) Control sludge without AgNP (CR) and b) Control sludge with AgNP (CR_{AgNP}).

Production of VLB-EPS and LB-EPS were almost equal in CR and CR_{AgNP} sludges. The main difference was observed in TB-EPS production as shown in Figure 6.44. At the end of Phase II, VLB-EPS production was greater than LB-EPS. At the same time, the least TB-EPS was observed in $CR_{AgNP.}$ Probably, VLB-EPS was formed due to lysis of biomass. At the same time, an obvious difference was observed between the sludges in terms of MLVSS, that were about 1905 mg/L and 3250 mg/L in CR_{AgNP} and CR, respectively (Figure 6.41). In the middle of Phase II at about 80 days of operation, the dramatic decrease in MLVSS indicates inhibition. Some of biomass was negatively affected in the presence of AgNP. The same observation was also made in respirometric tests conducted with these sludges as shown in Figure 6.42. A slight decrease in OUR was observed in the presence of AgNP. In this phase, in the CR_{AgNP} reactor AgNP existed probably still in ionic form (Ag^{+}) and was not agglomerated yet.

Compared to RP sludge, CR sludge was fed with lower amounts of peptone. Peptone has a higher complexation capacity with Ag^{+} than other organics found in CR feed (Feed 1). In this dissertation, the free Ag^{+} was probably at higher levels in CR_{AgNP} sludge. Therefore, a slight inhibition was observed in CR_{AgNP} sludge compared to RP_{AgNP} sludge. A previous study (Çeçen and Kılıç, 2015) indicated that the inhibitory effect of Ag^{+} was very low in RP sludge due to complexation with peptone.

With increasing dose of AgNP in CR_{AgNP} sludge, there was particularly an increase in TB-EPS production compared to CR. On the other hand, VLB-EPS and LB-EPS were produced almost at the same level in both reactors. However, in the last phase (Day 138) a steady increase was observed in all EPS fractions, in particular in VLB-EPS and LB-EPS. Parallel experiments have shown that also the surface of CR_{AgNP} sludge became slightly more negative while hydrophobicity was slightly decreased. However, these changes shown in Figure 6.45 were not significant. Statistical analysis showed that surface charge (p>0.05) and hydrophobicity (p>0.05) were not different in CR and CR_{AgNP} sludges. On the other hand, in RP and RP_{AgNP} sludges, there were significant differences with respect to surface charge (p<0.05) and hydrophobicity (p<0.05) of sludges. Results showed that, the type of organic carbon fed to a sludge had an effect on surface properties of sludges both in the absence and presence of AgNP.

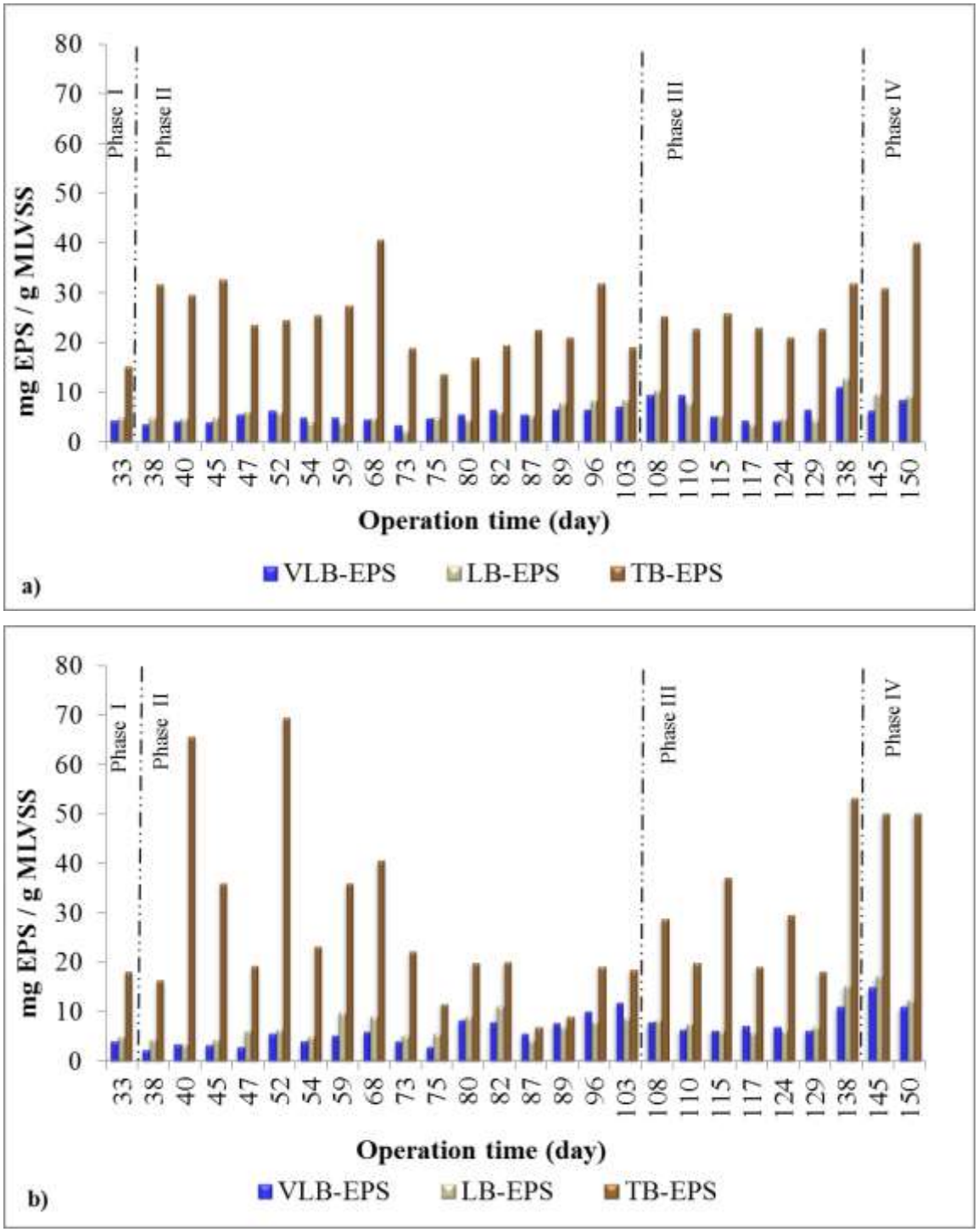


Figure 6.44. Fractionation of EPS in a) Control sludge without AgNP (CR) and b) Control sludge with AgNP (CR_{AgNP}).

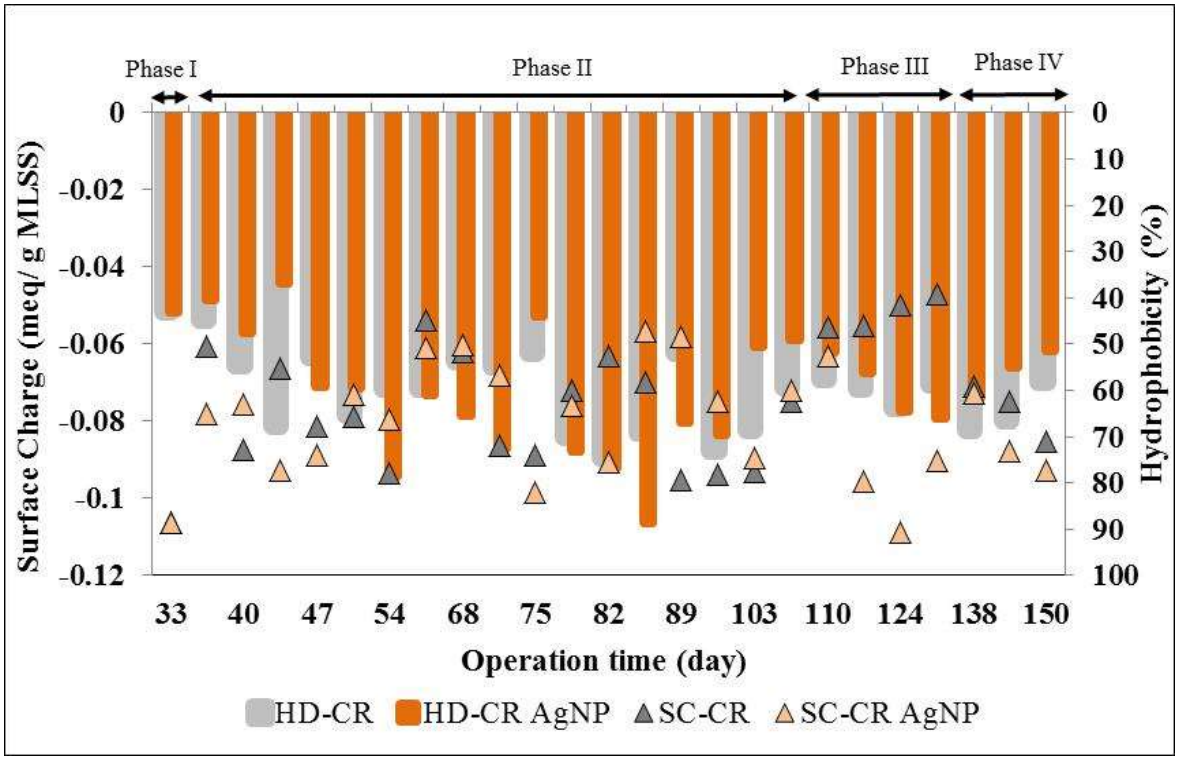


Figure 6.45. Sludge surface charge (SC) and hydrophobicity (HD) (CR:Control sludge without AgNP, CR_{AgNP}: Control sludge with AgNP).

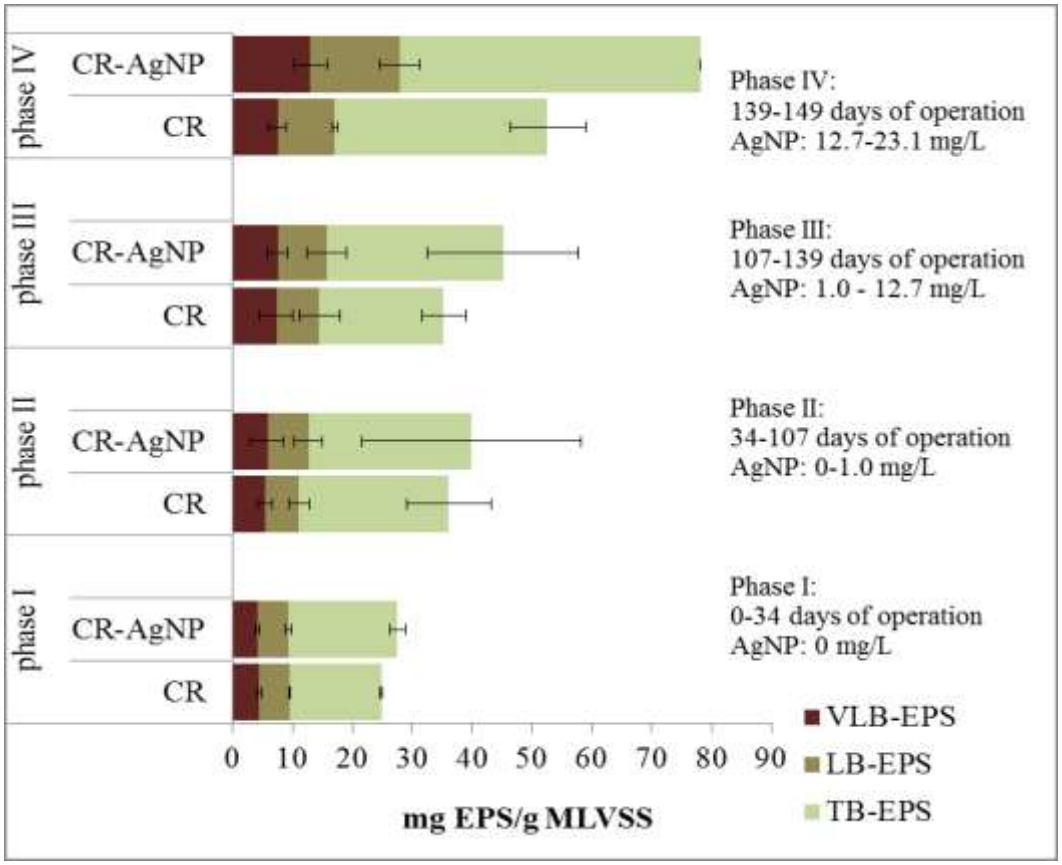


Figure 6.46. Fractionation of EPS in different phases of CR and CR_{AgNP} operation (n=26) (VLB-EPS: Very Loosely Bound-EPS, LB-EPS: Loosely Bound-EPS, TB-EPS: Tightly Bound-EPS).

6.3.3.7. Changes in the Molecular Weight of Protein-EPS. During operation of CR and CR_{AgNP}, EPS were analyzed by HPSEC. The data presented in Figure 6.47 shows the MW of proteins and reflect the results of about 285 measurements.

In Phase I, CR_{AgNP} did not receive any AgNP. MWs of Protein-EPS in this sludge were lower than 9.2 kDa. Proteins with higher MWs were observed in TB-EPS fractions only (Figure 6.47).

In Phase II, both in CR and CR_{AgNP} sludges, in Sp-EPS and VLB-EPS fractions smaller proteins were dominant. In CR sludge, three types of proteins were produced in the LB-EPS fraction. On the other hand, five types of proteins were detected in CR_{AgNP} sludge. It is thought that new proteins were produced in the MW ranges of 570-650 and 9.2-16 kDa in the presence of AgNP. In this phase, AgNP concentration was in the range

of 0.15-1.0 mg/L. CR_{AgNP} sludge could tolerate 1.0 mg/L of AgNP by producing new proteins in LB-EPS. According to colorimetric results shown in Figure 6.46, in Phase II, there was a slight difference between CR and CR_{AgNP} sludges in terms of LB-EPS production.

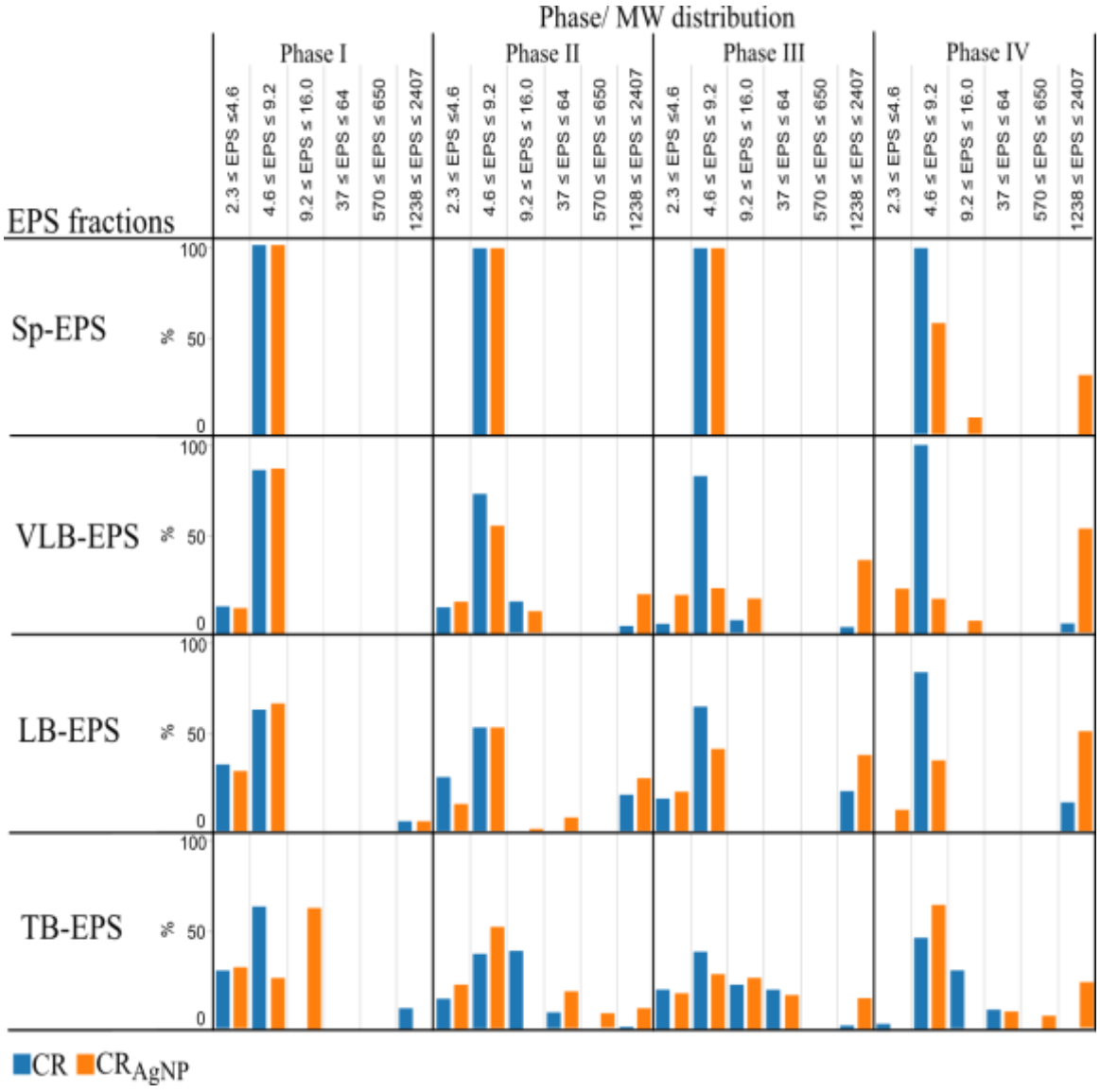


Figure 6.47. MW of EPS in different phases of CR and CR_{AgNP} operation.(VLB-EPS: Very Loosely Bound-EPS, LB-EPS: Loosely Bound-EPS, TB-EPS: Tightly Bound-EPS).

In Phase III, the same proteins were produced in both reactors. However, the percentage of each protein type was different. For example, in CR sludge smaller proteins were dominant, but in CR_{AgNP} higher proteins dominated as shown in Figure 6.47. Proteins having higher MWs in VLB-EPS, LB-EPS and TB-EPS fractions were produced in higher amounts in CR_{AgNP} sludge. In the presence of AgNP, probably cell lysis occurred leading to such biomass associated products (BAP).

In Phase IV, the greatest difference between the reactors was seen in Sp-EPS where higher MW of proteins ($1238 \leq EPS \leq 2407$) were observed first time in Sp-EPS in CR_{AgNP} sludge. In other fractions, MWs of proteins were approximately the same, but the percentage of proteins was different. It was thought that microorganisms protected themselves from inhibitory effects of AgNP by regulating the amount of proteins in EPS.

Table 6.6. Comparison of sludges: Control without AgNP (CR), with AgNP (CR_{AgNP}); Peptone without AgNP (RP), with AgNP (RP_{AgNP})

Reactor	**Feed Characteristic**	**AgNP**	**Protein-EPS (mg EPS/g MLVSS)**	**Carbohydrate-EPS (mg EPS/g MLVSS)**	**VLB-EPS (mg EPS/g MLVSS)**	**LB-EPS (mg EPS/g MLVSS)**	**TB-EPS (mg EPS/g MLVSS)**	**MW characteristics of protein-EPS**
RP	Proteinaceous feed	-	23.8±6.9	4.2±1.9	4.6±0.9	3.6±0.7	20.0±7.4	Most of protein had MW of <8.8 kDa. Up to 5 different proteins were observed.
CR	Carbonaceous feed	-	28.5±6.0	8.9±3.8	6.1±1.9	6.3±2.4	25.4±6.8	Most of protein had MW of <9.2 kDa. Up to 5 different proteins were observed especially in TB-EPS.
RP_{AgNP}	Proteinaceous feed	+	29.9±11.0	7.3±6.2	6.7±2.7	6.4±2.9	24.4±12.8	New proteins were produced in the MW ranges of 28-40, 40-70, 576-1571 and 1571-2241 kDa in the presence of AgNP.
CR_{AgNP}	Carbonaceous feed	+	34.3±16.2	9.5±4.7	6.7±3.1	7.8±3.4	29.3±16.8	New proteins were produced in the MW ranges of 9.2-16, 37-64, 570-650 and 1238-2407 kDa. Sp-EPS had higher MW indicating higher cell lysis.

7. CONCLUSIONS

The first part of the study showed that EPS production depended on the COD/TKN ratio in the feed. In reactors receiving organic carbon, a stable EPS composition was reached only in long-term operation although steady-state conditions were soon achieved regarding substrate removal. Monitoring stabilization of EPS is of importance since EPS affect sludge behavior as well as the response of sludge to toxics. The results suggest that EPS would be more rapidly stabilized in tertiary nitrification or in the case of lower organic loadings.

Although the COD/TKN ratio did not seriously affect the total EPS production, the composition of EPS, namely the relative proportion of proteins and carbohydrates, was evidently affected by this ratio. In all sludges EPS were dominantly composed of proteins. However, the EPS of nitrifying sludge receiving no organic carbon (COD/TKN= 0) had a definitely higher protein content than others. The most striking result was that this sludge produced as much total EPS per biomass as others receiving also organic carbon. Further, regardless of the COD/TKN ratio, in all sludges tightly bound EPS prevailed.

In all sludges, most of the proteins (about 90 %) had relatively low MWs ≤16 kDa. Wet analyses gave a rough idea on EPS composition in terms of carbohydrate and proteins. On the other hand, HPSEC measurements provided a detailed insight into protein-EPS and showed that some proteins shifted from tightly bound fractions to others.

In the second part of the study, the composition and fractionation of EPS and physicochemical properties of activated sludges were compared when fed with different organic substrates. In long-term operation of each reactor at steady-state, total production of EPS depended on the type of organic substrate in the feed. However, more important was the change that took place in EPS composition, namely the relative proportion of proteins and carbohydrates. Again, in all sludges EPS were dominantly composed of proteins even if proteins were absent in the feed. However, in the sludge receiving a proteinaceous feed, the protein to carbohydrate (P/C) ratio was clearly higher.

Composition of EPS evidently affected also sludge hydrophobicity and surface charge. Surface properties are of particular interest from the point of engineering. A highly hydrophobic sludge, as the one fed with peptone in this study, will be susceptible to sorption of organic xenobiotics. On the other hand, sludges receiving a mixture of peptone, glucose and acetate, or glucose alone had more negative surface charges and were less hydrophobic. Therefore, such sludges are likely to retain soluble species like metal ions or their complexes.

HPSEC measurements gave detailed information about protein-EPS. The variety and size of proteins were clearly different in the case of a proteinaceous feed. At the other extreme, in the absence of proteins in feed, as in the case of glucose feed, the proteins found in different bound EPS fractions are thought to be utilized by biomass.

For practical purposes, the results presented in this part of dissertation indicate that differences will arise in EPS and surface characteristics of an activated sludge depending on the composition of a wastewater. The results would be of interest to all readers involved with biological treatment. EPS and surface characteristics might affect uptake of xenobiotics such as metals and organics, sludge settling, dewatering and membrane fouling in full-scale treatment systems.

Short-term respirometric experiments showed that AgNP was less toxic than Ag^+ ion. After exposure to Ag^+, microorganisms produced more looser EPS fractions (VLB-EPS and LB-EPS), probably to diminish the inhibitory effect of silver. Moreover, voltammetric results revealed that the very loosely bound EPS (VLB-EPS) fraction could bind more Ag^+ and AgNP than other fractions. LB-EPS and TB-EPS fractions had a tendency to complex more with AgNP than the Ag^+ ion. Moreover, this complexation was highly dependent on the MW of proteins.

Long-term experiments in semi-continuously fed batch reactors showed that even at high concentrations of AgNP, this nanometal did not have a negative effect on organic carbon removal, because it lost its stability. Therefore, in a real WWTP, inhibition will not be observed. However, with time AgNP might be accumulated in biomass. Therefore,

sludge disposal may become an important problem when sludges are used as soil amendment.

Besides, long-term experiments showed that EPS production and fractionation depended on AgNP dose. The amount of EPS fractionation differed between reactors. Moreover, different proteins were produced in the presence of AgNP in each different activated sludge system.

This dissertation highlighted that EPS are capable of binding silver ions (Ag^+) as well as silver nanoparticles (AgNP). Both types of silver were complexed with EPS and entered the solid phase. An important finding of this study was that a new fraction of EPS was identified that was not mentioned previously in literature. This fraction was named as very loosely bound EPS (VLB-EPS). VLB-EPS were shown to play an important role in binding metals. Most of Ag^+ and AgNP was complexed with this fraction. However, this very loosely bound fraction can easily become soluble. Therefore silver species can be released to receiving waters.

In literature, most of AgNP studies are performed in a short-term period. To our knowledge, such a long-term study has not been performed yet. Short-term and long-term exposure of AgNP to activated sludge gave different results. It is thought that in a real wastewater treatment system low levels of AgNP will not exert a drastic inhibitory effect in long term. Besides, at studied AgNP concentrations, substrate degradation will not be inhibited. However, AgNP had an effect on EPS production. Prolonged exposure to AgNP led to an extreme production of each EPS fraction. It is believed that bacteria protected themselves from inhibitory effects of AgNP by regulating their EPS production.

8. RECOMMENDATIONS FOR FUTURE WORK

In view of the findings of this study, the following recommendations are made for future researches:

1) Only a limited number of studies found in literature focus with nitrifying sludges. In the present study, EPS production capacity of a nitrifying sludge was examined in detail. However, the effect of nanoparticles on nitrifying sludge and on EPS production needs to be studied in long term.

2) For better understanding of EPS dynamics, molecular microbiology techniques might be integrated into experiments to examine the relationship between EPS and microbial ecology.

3) Theoretical speciation calculation showed that most of Ag^{+} was bound to biomass. Nowadays, recovery of precious metals from every kind of waste is a very hot topic. Recovery of metals can be incorporated into inhibition studies.

4) PVP coated AgNP was used in experiments and its inhibitory effect was investigated in short- and long-term periods. Further investigation is needed to characterize the surface of AgNP and to elucidate whether bacterial growth is sensitive to surface charges or not.

5) In this study, inhibitory effects of Ag and AgNP on substrate removal and EPS production were examined. In real cases, many different metals can coexist in a wastewater. EPS production in the presence of different metals is another point requiring additional research. Due to the complex nature of wastewater, heavy metals can exist in the presence of other toxic compounds or complexing agents. Therefore, the inhibitory characteristics of metals may change. This is a point that should also be investigated.

6) In this study, EPS (biopolymer) production was examined in different activated sludges that are composed of mixed cultures. However, there are some studies about the stimulation of EPS production in pure cultures. The results in the present study showed that feed composition was very important in EPS production. More research is needed to stimulation of EPS production in pure cultures since these biopolymers can be used in many industrial applications including wastewater treatment, food products, drug delivery, medical adhesives, vaccines.

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