UNDERSTANDING DEFLOCCULATION OF ACTIVATED SLUDGE UNDER TRANSIENTS OF SHORT-TERM LOW DISSOLVED OXYGEN

by

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ABSTRACT

Deflocculation is a common upset event in biological wastewater treatment plants and causes significant problems in biosolids discharge and environmental management. However, fundamental understanding of deflocculation is limited. The overall objective of this work was to explore the fundamentals for deflocculation under transients of short-term low dissolved oxygen (DO). The investigation was carried out in a sequence of batch and continuous experiments on activated sludge, followed by batch experiments on *E. coli* suspensions.

Both batch and continuous experiments on activated sludge demonstrated deflocculation of bioflocs under the transients of low DO (< 0.5 mg/L). Under the short-term low DO (in hours), turbidity increased by 20 times in the batch system and by 1-2 times in the continuous system, concentrations of suspended solids increased by 1-2 times, number of small particles (< 12.5

μm) increased by 2 times, more soluble EPS (proteins and humic substances) were released into supernatant or treated effluents, the removal efficiency of organic compounds was reduced by 50-70%. A 40% of increase in bulk K^+ but a 30% of decrease in bulk Ca^{2+} under the DO limitation were observed in the batch experiments. There were significant increases in bulk K^+ and decreases in bulk Ca^{2+} in the continuous experiments. Reversible changes were observed within 24 hours once the DO stress was removed. Floc strength of the remaining bioflocs after deflocculation increased. Deflocculation under the short-term low DO was consistent with an erosion process. The addition of selected chemicals (i.e., Ca^{2+}, tetraethylammonium chloride, glibenclamide, and valinomycin) did not prevent deflocculation under the short-term low DO.

It is proposed that a DO stress causes an efflux of cellular K^+ but an influx of extracellular Ca^{2+}, resulting in a decreasing ratio of Ca^{2+}/K^+ in extracellular solution and thereby causing deflocculation. The *E. coli* tests supported that increasing bulk K^+ under the DO limit was due to the release of cellular K^+ and was a stress response to the DO limitation.

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PREFACE

This research is to enhance the understanding of deflocculation of biosolids due to transient conditions. Five publications have been or will be produced, covering literature review, experimental results and discussion from different research stages. The layout of each publication is as follows:

- "Exploring Mechanisms for Disintegration of Bioflocs under Stresses: The Roles of Extracellular Proteins and Polysaccharides in Cell Aggregation and Microbial Responses to Environmental Stresses", to be submitted to *Critical Reviews in Environmental Science and Technology*, based on Sections 2.4 to 2.7;
- "The Effects of Short-term Dissolved Oxygen Transients on Activated Sludge", submitted to *Water Quality Research Journal of Canada*, based on Sections 4.1.1 and 4.1.2.2;
- "Strategies for Minimizing Deflocculation of Biosolids due to Oxygen Disturbances", *Water Science and Technology*, 55(6), 173-180, based on Sections 4.2.1 and 4.2.3.
- "Role of Cellular Cation Fluxes in Response to Disturbances of Short-term Low Dissolved Oxygen on Activated Sludge", to be submitted to *Water Environment Research*, based on Sections 4.1.1, 4.2.1, 4.3.1 and 5.2.
- "Effect of Dissolved Oxygen Variations on Shear Sensitivity of Bioflocs", being drafted (co-author with Olive Yuan), to be submitted to *Water Research*, part of this paper is based on Sections 4.2.2 and 4.2.3.

CHAPTER 1 INTRODUCTION

1.1 Research Statement

1.1.1 Motivation of the Present Study:

Transients, or unsteady state conditions, often cause problems in solid-liquid separation in aerobic biological wastewater treatment plants. Some of the common transients occurring in the treatment plants include fluctuations in organic loading (Majone et al., 1999; Pernelle et al., 2001), low dissolved oxygen (DO) (Starkey and Karr, 1984; Wilén and Balmér, 1998, 1999; Wilén et al., 2000a, b), temperature variations (Lapara et al., 2001; Morgan-Sagastume and Allen, 2003), and toxicant overloading (Neufeld, 1976; Galil et al., 1998; Bott and Love, 2002). As a typical aerobic biological treatment process, activated sludge systems are often exposed to the transients, which affect biosolids separation in secondary clarifiers. The poor settling performance leads to undesirable removal of organic compounds, loss of biosolids and toxicant carry-over.

The most common settling problems are deflocculation, pin-point flocs, bulking and scum/foaming. Deflocculation is a dissociation of aggregated biomass particles, imposing a high concentration of suspended solids (SS) in treated effluents. Pin-point flocs are small flocs that can not flocculate well, causing turbid effluents (Jenkins et al., 1993; Rittmann and McCarty, 2001). Bulking refers to an overabundance of filamentous bacteria in the activated sludge, resulting in less settling sludge and a high sludge volume index (SVI) (Metcalf and Eddy Inc., 1991; Jenkins et al., 1993; Rittmann and McCarty, 2001). The formation of scum or foam is mainly due to the presence of non-degradable surfactants or specific microorganisms,

such as *Norcardia sp.* and *Microthrix sp*. (Rittmann and McCarty, 2001). Scum or foaming often causes a loss of biosolids. Among these settling upsets, deflocculation is the least studied.

Transients of short-term low DO (< 1 mg/L), often arising from organic overloading or insufficient aeration, cause deflocculation of activated sludge. Long-term (days or weeks) DO limitations have a different impact from short-term (hours) DO limitations. It has been known that filamentous bulking is a general consequence of a long-term DO limitation (Jenkins et al., 1993; Rittmann and McCarty, 2001; Gaval and Pernelle, 2003). In contrast, deflocculation of activated sludge occurs under a short-term low DO level, as characterized by a high turbidity in treated effluents (Starkey and Karr, 1984; Wilén and Balmér, 1998, 1999; Wilén et al., 2000a, b).

Besides increases in turbidity and SS in effluents, loss of bio-catalysts and carry-over of toxicants into treated effluents due to the deflocculation of biosolids are of significant concern in industry. Biosolids are the catalysts in aeration tanks, responsible for removing organic contaminants from incoming wastewater. The loss of biosolids represents the loss of bio-catalysts in the aeration tanks. In addition, toxicants attached or absorbed onto biosolids are prone to be carried over into treated effluents during deflocculation, giving rise to the issue of toxicity. All of these lower the overall operating performance of treatment plants.

Currently, despite the fact that deflocculation is a common upset event in treatment plants, fundamental understanding of deflocculation is limited. Effective strategies for minimizing the deflocculation are not available. Bioflocs are mainly composed of various bacteria held by extracellular polymeric substances (EPS) and inorganic matter. It is generally recognized that EPS and divalent cations, such as Ca^{2+} and Mg^{2+}, play an important role in stabilizing biofloc

matrix and improving bioflocs' settleability (Li and Ganzarczyk, 1990; Bura et al., 1998; Liss, 2002; Higgins and Novak, 1997b, c). Therefore, an in-depth understanding of functional roles of EPS components and cations in aggregation of bioflocs will facilitate the fundamental understanding of deflocculation. This will contribute to the development of effective strategies for maintaining a desirable operating performance under transient disturbances.

1.1.2 Objectives

The overall objective of this research is to enhance the understanding of deflocculation of activated sludge under transient conditions, particularly, under a short-term low DO. Specific objectives are:

I. To assess the effects of short-term low DO (< 0.5 mg/L for 6 hours) on removal of organic compounds, solids discharge, and properties of bioflocs;

II. To quantify the extent of deflocculation under the DO transients;

III. To understand the fundamentals for deflocculation under the DO transients;

IV. To explore strategies for minimizing the deflocculation due to the transient conditions;

1.1.3 Hypotheses

Hypotheses held in this research are:

i) Transients of short-term low DO cause deflocculation of activated sludge;

ii) Deflocculation under the short-term low DO is reversible;

iii) Deflocculation due to short-term DO limit is caused by: (a) an excretion or activation of extracellular enzymes to degrade EPS proteins; and/or, (b) a decreasing ratio of divalent to monovalent ions in extracellular solution.

1.1.4 Overall Approaches

Overall, three experimental stages were performed to examine and to understand deflocculation of activated sludge under transients of short-term low DO:

I. Batch experiments using fresh municipal activated sludge were conducted in four parallel batch reactors, to investigate the effects of short-term low DO on changes in turbidity, SS, EPS components and extracellular cations. In addition, enzymatic tests were performed to elucidate the importance of proteins and polysaccharides in bioflocs' flocculation. A preliminary assessment on the mechanisms for deflocculation was achieved at this stage.

II. Continuous experiments were performed over 5 months in four parallel sequencing batch reactors (SBRs) fed with pulp and paper effluents, to examine deflocculation and impacts of short-term DO variations on treatment performance. The mechanism for sludge deflocculation was proposed and strategies for deterring the deflocculation were tested;

III. Batch tests on *E. coli* cells under the DO limitation were performed to examine changes in extracellular cations in the *E. coli* suspensions upon the O_2 deficits, and to support the mechanism understanding of deflocculation.

1.2 Industrial Significance

This study presents a detailed identification of the impacts of short-term low DO on activated sludge. This work examined the DO impacts on removal of organic compounds (characterized by soluble chemical oxygen demand, i.e., SCOD), effluent quality and physical properties of bioflocs, including turbidity, effluent suspended solids (ESS), concentrations of organic compounds, particle size distribution and floc strength. The results will provide a detailed description of the DO impacts on aerobic wastewater treatment, as well as a sound basis for understanding deflocculation.

Negative impacts of deflocculation on operating performance are of significant concern. It has been known that deflocculation lowers overall treatment performance by causing a loss of biosolids, toxicant carry-over and a poor effluent quality. It is of importance to study and understand deflocculation, so as to better control solid-liquid separation responding to disturbances.

This research enhances the mechanism understanding of deflocculation. To date, a cause-and-effect understanding of deflocculation has not been well established. This work is to explore what triggers the deflocculation, and to provide a link between cellular stress response and macroscopic effects observed in industrial treatment systems. An in-depth understanding of deflocculation will aid in developing effective operating strategies for minimizing or preventing deflocculation due to disturbances, and will also contribute to other relevant biological processes, such as fouling control in biofilms.

1.3 Thesis Outline

A background introduction of the research topic, objectives and hypotheses is presented in Chapter 1. An overview of literature is presented in Chapter 2, as the basis for a publication on reviewing the functional roles of EPS proteins and polysaccharides, and microbial stress responses. Overall experimental approaches to investigate deflocculation under transients of short-term low DO were addressed in Chapter 3, including batch experiments using fresh municipal activated sludge, continuous experiments using wastewater from a bleached kraft pulp and paper mill, and batch tests using *E. coli* suspensions. The experimental results from each stage are delineated in Chapter 4, providing a basis for four publications on the DO effects in batch and continuous systems, fundamental understanding of deflocculation, and the DO impacts on structure and strength of bioflocs, respectively. An overall discussion is addressed in Chapter 5. Overall conclusions drawn from this work are presented in Chapter 6, followed by engineering and scientific significance of this work in Chapter 7. Recommendations for future work are discussed in Chapter 8.

As described previously, this thesis mainly consists of five publications submitted to refereed journals. A review paper was produced based on Chapter 2. To keep the thesis coherent and concise, contents of other four publications on identifying and understanding deflocculation have been spread throughout Chapters 3 to 6.

CHAPTER 2 LITERATURE REVIEW

This chapter reviews studies on transients, formation of bioflocs, functional roles of EPS proteins and polysaccharides in cell aggregation, and cell stress responses to environmental disturbances. Sections 2.4 to 2.7 provide a basis for a review paper submitted to *Critical Reviews in Environmental Science and Technology*.

2.1 Biological Wastewater Treatment

In industry and municipality, wastewater is commonly treated by aerobic biological treatment processes, among which activated sludge process is widely used. An activated sludge system mainly consists of an aeration tank and a clarifier. In the aeration tank, microorganisms, in the presence of oxygen, consume organic contaminants in incoming wastewater. The organisms form bioflocs (e.g., biosolids) in the aeration tank. In the following clarifier, the bioflocs are separated from clean treated wastewater by gravity settling. Part of the settled bioflocs will return to the aeration tank for maintaining an optimal concentration of the organisms. The clean treated wastewater will be discharged from the top of the clarifier. Though treatment plants are designed based on steady-state conditions, transient conditions are common. Any disturbances to the operation of the aeration tank or the clarifier will have an influence on treatment efficiency and quality of treated wastewater.

2.2 Transients in Biological Wastewater Treatment

2.2.1 Overloading of Organic Substrates

Upon changes in feedstocks, overloading of organic substrates is often present in treatment plants, but is of less concern in aerobic treatment provided that sufficient aeration is supplied.

Organic overloading generates a concentration shock in organic nutrients, which are commonly measured as biochemical oxygen demand (BOD) or chemical oxygen demand (COD). Overall BOD/COD changes originate from variations in one or multiple organic components, which may impose a complicated system response. Thus, in order to simplify the problems, most current studies on organic transients use synthetic substrates rather than real wastewater. Storage response is a typical physiological adaptation of microorganisms to the overloading of BOD/COD, as characterized by a synthesis of storage polymers, such as glycogen and polyhydroxybutyrate (Daigger and Grady, 1982; Majone et al. 1999). Regarding solid-liquid separation under the substrate overloading, no other settling problems have been described except filamentous bulking under a combining effect of BOD overloading and oxygen deficits (Pernelle et al., 2001).

Compared to aerobic treatment, anaerobic systems are more sensitive to the substrate overloading and exhibit undesirable performance under the disturbance. Paula Jr. and Foresti (1992) examined the overloading effect of synthetic COD on performance of an upflow anaerobic sludge blanket reactor. A gradual decrease in methane production was observed, as well as a decreasing overall removal efficiency of COD. Xing et al. (1997) undertook a study on long-term (400 days) periodic variations in glucose concentrations (16 g/L in 3-day followed by 0 g/L in another 3-day). The results showed significant increases in effluent COD and decreases in methane production during the perturbation period.

2.2.2 DO Limitation

The impacts of low DO on solid-liquid separation have been identified. Low DO is one of the common process upsets (Berthouex and Fan, 1986; Archibald and Young, 2004). A long-

term (days or weeks) DO limitation has a different impact from a short-term (hours) DO limitation. It has been known that filamentous bulking is a general consequence of a long-term DO limitation (Jenkins et al., 1993; Rittmann and McCarty, 2001; Gaval and Pernelle, 2003). In contrast, deflocculation has been observed in the studies of short-term low DO, as mainly characterized by increases in effluent turbidity. In a continuous experiment fed with synthetic feeds, Starkey and Karr (1984) observed increasing supernatant turbidity after stopping air-flow for 10 hours (DO = 0.4 ± 0.2 mg/L). In a batch system under alternative aerobic (2 hours of air aeration) and anaerobic conditions (3-4 hours of N_2 purging), Wilén and co-investigators (Wilén and Balmér, 1998, 1999; Wilén et al., 2000a, 2000b) observed increases in supernatant turbidity (by 2-4 times) and increasing amounts of small flocs (2-20 μm) in the anaerobic cycles. All these results suggest a different system response to short-term low DO in comparison to a long-term low DO effect.

With respect to physicochemical properties of bioflocs, studies on the impacts of steady-state low DO showed that amounts of proteins and carbohydrates decreased (Nielsen et al., 1996; Sponza, 2002) and cell surfaces became less hydrophobic (Palmgren et al, 1998). However, currently, little is known about the impacts of DO transients on the physicochemical properties of bioflocs.

2.2.3 Temperature Variations

Temperature variations have been shown to lower the engineering performance of treatment plants. Temperature transients are due to upstream operating variations or seasonal changes. Using pharmaceutical wastewater, Lapara et al. (2001) found a declining removal rate of soluble COD (SCOD) and a reduction in microbial population diversity upon the temperatures

increasing from 30ºC to 70ºC. Similarly. Morgan-Sagastume and Allen (2003) observed a reduction in the removal rate of SCOD, increases in supernatant turbidity and ESS, a high SVI and an occurrence of deflocculation when the system was exposed to a 15ºC upshift from 30ºC to 45ºC. Further analysis on microbial communities showed that the structure of bacterial communities diverged upon the upshift from 30ºC to 45ºC (Nadarajah et al., 2007).

Different from the temperature upshifts, limited studies are available on the influence of temperature downshifts. Industrial wastewater facilities may experience a substantial temperature drop in the wintertime. Wilén et al. (2000a) studied a temperature downshift to 4ºC using municipal activated sludge, and observed deflocculation as characterized by increasing turbidity. The authors hypothesized that a decrease in microbial activity under the low temperature might be the reason for deflocculation but without further proving.

2.2.4 Toxicant Transients

The fluctuation of toxic chemicals from upstream feedstock or operating accidents is another transient occurring in treatment plants. Apart from the above substrate transients (organic and DO), changes in feed compositions can also be from toxicant transients, that is, concentration changes in specific chemicals that are toxic to microorganisms, such as heavy metal (Neufeld, 1976), phenol (Galil et al., 1998) and electrophilic compounds (Bott and Love, 2002, 2004). Deflocculation of biosolids was observed in studies on various toxicant shocks, but different mechanisms were proposed.

Phenol transients may induce a lethal shock on biomass, rather than a physiological impact. Using municipal sludge, Galil et al. (1998) studied the effects of phenol overloading by measuring turbidity and polysaccharide concentrations in the supernatants after 30-min. of

gravity settling, as well as oxygen uptake rate (OUR). A high turbidity and an increase in the polysaccharide level were observed. The OUR was immediately reduced under the phenol shock. Schwartz-Mittelmann and Gali (2000) suggested that the membrane damage by the phenol shock was the primary reason for deflocculation.

Bott and Love (2002) investigated deflocculation under transients of toxic electrophilic chemicals and proposed a different hypothesis compared to the above phenol shocks. Electrophilic chemicals (i.e., electrophiles) are those reactive species that are electron-deficient and tend to attract electrons during chemical reactions, such as H^+, NO_2^+ and $C_6H_5N_2^+$. The authors observed deflocculation of activated sludge under a shock loading of N-ethylmaleimide (NEM) and chloro-2,4-dinitrobenzene (CDNB). They put forward a glutathione-gated potassium efflux (GGKE) mechanism. Based on this mechanism, electrophilic chemicals react with glutathione in bacteria to form glutathione-electrophile adducts (GSX). The GSX activates a potassium efflux channel, to release K^+ from cytoplasm into extracellular solution. As a consequence, the ratio of monovalent to divalent ions increases in the extracellular surrounding, causing deflocculation.

2.3 Understanding of Bioflocs

Aggregation of various microbial species, formed as bioflocs, is important in maintaining a desirable performance in many aerobic biological wastewater treatment plants. Various microorganisms are mainly connected through a bonding of EPS and inorganic cations. Different forces, including van der Waals force, hydrophobic, EPS polymer bridging and electrostatic force, are involved in the aggregation of bioflocs (Liss, 2002). Upon exposure to environmental disturbances (e.g., temperature, DO), bioflocs are prone to disassociate from

each other, a process known as deflocculation. This will result in a poor quality of treated effluents and lower overall performance of treatment plants.

Regarding the structure of bioflocs, a two-layer structure has been recognized in recent studies. Eriksson and Alm (1991) proposed the structure of bioflocs as an inner layer surrounded by an outer layer. Liao and co-workers (Liao, 2000; Liao et al., 2002) supported the notion of a two-layer structure of bioflocs. They further proposed that dominant forces in the outer layer and inner layer were different: electrostatic and ionic interactions, as well as hydrogen bonds, were mainly involved in the outer layer. In comparison, van der Waals and hydrophobic forces were dominant in the inner layer. Sheng et al. (2005) concluded that the outer region of bioflocs accounts for nearly 20% of total bioflocs' mass and the presence of this region lowers the overall stability of bioflocs.

Different mechanisms for formation of bioflocs have been proposed, including alginate theory, Derjaguin and Landau and Verwey and Overbeek (DLVO) theory and divalent cation bridging (DCB) theory. The alginate theory involves formation of alginate gels from alginate and Ca^{2+}. However, recent studies show that adding Mg^{2+} can stabilize the floc matrix, similar to Ca^{2+} (Higgins and Novak, 1997b, c; Sobeck and Higgins, 2002; Morgan-Sagastume and Allen, 2005). This challenged the validity of the alginate theory. According to the DLVO theory, either monovalent or divalent cations should be able to promote floc formation, which contradicts to the notion of negative impacts of monovalent ions on floc stability and settling properties (Higgins and Novak, 1997a-c; Sobeck and Higgins, 2002). In comparison, the DCB theory for aggregation of bioflocs has been suggested in recent research (Biggs et al., 2001; Sobeck and Higgins, 2002). In the DCB theory, divalent ions, such as Ca^{2+} and Mg^{2+}, can

bridge negatively charged functional groups in EPS to facilitate flocculation of bioflocs. Accordingly, both EPS and divalent cations play important roles in the formation and stabilization of bioflocs.

Evidence shows that divalent cations (e.g., Ca^{2+} and Mg^{2+}) and monovalent cations (e.g., Na^{+} and K^{+}) have different influences on floc stability. From batch and continuous experiments, Higgins and co-works (Higgins and Novak, 1997a-c; Sobeck and Higgins, 2002) highlighted the importance of Ca^{2+} and Mg^{2+} in facilitating sludge flocculation and improving the settling and dewatering properties of activated sludge. In contrast, monovalent cations could trigger the release of biopolymers into surrounding solution and diminish the floc strength (Higgins and Novak, 1997a-c; Murthy and Novk, 2001). As for monovalent ions, however, some experimental results indicate that the effect of K^{+} on floc stability is different from that of Na^{+}. Murthy and Novak (1998) stated that with increasing [K^{+}], SVI decreased, and effluent turbidity declined first and then increased. An examination of [Na^{+}] did not demonstrate the similar trend. Müller et al. (2002) also found that concentrations of ESS decreased first before increasing at elevated concentrations of K^{+}. Overall, compared to monovalent cations, divalent cations play a positive role in flocculation of bioflocs.

In addition to divalent and monovalent cations, the important role of trivalent cations, especially Fe^{3+}, in flocculation has been emphasized by Rasmussen and Nielsen (1996), Keiding and Nielsen (1997), Nielsen and Keiding (1998) and Park et al. (2006a, b). Due to its high valence, Fe^{3+} was able to form a more stable bond with EPS compounds than divalent cations (e.g., Ca^{2+}). Accordingly, removal of Fe^{3+} via biological or chemical reduction of Fe^{3+} to Fe^{2+} could lead to deflocculation of sludge.

The amount of Fe in sludge varies significantly, from 1 to above 100 mg Fe/g VS, depending on types of incoming wastewaters, treatment processes, and types of sludge (Park et al., 2006b). Due to domestic activities, municipal wastewaters tend to contain a higher level of Fe than industry wastewaters. In addition to secondary treatment (e.g., the activated sludge process), some treatment plants have tertiary treatment to further remove nutrients, such as nitrogen and phosphorus, or to further improve solid/liquid separation by adding coagulants. Concomitantly with biological removal of phosphorus, some treatment plants externally add Fe to chemically remove phosphorus (Rasmussen and Nielsen, 1996; Keiding and Nielsen, 1997). In promoting the solid/liquid separation and sludge dewatering, some treatment plants use iron salts as a coagulant (Deneux-Mustin et al., 2001; Buzier et al., 2006). All of these will lead to a high level of Fe accumulated in waste sludge for digestion treatment. Depending on the location of Fe addition, some of the Fe may end up in the return/waste sludge from secondary treatment. In general, the amount of Fe extracted from anaerobically stored sludge is 1-fold higher than that from fresh sludge (Rasmussen and Nielsen, 1996). In secondary treatment, the amount of Fe extracted from return/waste sludge is higher than that from fresh mixed liquor sludge. Wilén et al. (2004) reported approximately 60 mg Fe/g VS present in the return sludge from an activated sludge process. Bott and Love (2002) examined the Fe level in fresh municipal mixed liquor sludge, and found a range of 1-4 mg Fe/g MLVSS. Morgan-Sagastume (2003) analyzed the Fe level in fresh mixed liquor sludge fed with pulp and paper industry wastewater, and reported a level of 1-2 mg Fe/g MLVSS. Due to a wide range of Fe levels present in biological treatment systems, it is likely that the importance of Fe^{3+} in flocculation/deflocculation varies as well. Reduction of Fe^{3+} has been proposed to most likely

occur in anaerobic storage and anaerobic digester where the concentration of Fe is significant (Rasmussen and Nielsen, 1996).

Compared to removal of Ca^{2+}, removal of Fe^{3+} is proposed to cause a different type of deflocculation. Keiding and Nielsen (1997) and Nielsen and Keiding (1998) concluded that deflocculation caused by reduction of Fe^{3+} was a "dissolution" process of bioflocs. Using return activated sludge from a municipal treatment plant, the authors compared the impacts of removal of Fe^{3+} and removal of Ca^{2+} on the changes in surface charge density of bioflocs, measured by zeta potential. They observed a significant change in the surface charge of bioflocs by removing Ca^{2+} whereas an insignificant change by removing Fe^{3+}. Hence, removal of Ca^{2+} was proposed to mainly affect the surface of bioflocs. In contrast, removal of Fe^{3+} could lead to bioflocs' breakup.

In respect to reflocculation of activated sludge, the role of oxidation of Fe^{2+} to Fe^{3+} in triggering the reflocculation was found to be minor. Using municipal return activated sludge containing approximately 60 mg Fe/g VS, Wilén et al. (2004) examined the possible involvement of Fe in reflocculation. The sample first underwent a deflocculation process by purging with N_2, and then a reflocculation process by supplying O_2 or NO_3^- into the system. The authors examined the oxidation rates of Fe^{2+} to Fe^{3+} upon the addition of O_2 or NO_3^-. It was found that using O_2 or NO_3^- produced similar oxidation rates of Fe^{2+} to Fe^{3+}, but different extents of reflocculation: A more pronounced reflocculation occurred when O_2 was used.

In spite of considerable progress made on studying EPS, their functional role in the aggregation of bioflocs has not been well understood. This is mainly due to the complexity and

dynamics of EPS. The characteristics of EPS vary with types of substrates and operating conditions. A detailed review on the EPS of pure- and mixed-species is presented below.

2.4 Structure and Function of EPS

2.4.1 Structure of EPS

It should be noted that EPS have other names, such as exocellular or extracellular polymers (Pavoni et al., 1972; Starkey and Karr, 1984; Frølund et al., 1996), or exopolysaccharides (Davies et al., 1993; Burdman et al., 2000; Danese et al., 2000a). All of them refer to the same material, that is, polymeric material present outside cells or attached on cell surfaces.

Considerable progress has been made on the study of production, composition and properties of EPS. EPS are a mixture of polymers originating from microorganisms and incoming wastewater. They form a three-dimensional, gel-like matrix encasing microorganisms inside. Flemming and Wingender (2001) described the EPS matrix as a "house" for microorganisms. Such a "house" allows various consortia of microorganisms to live in a harmonious way and to develop a synergistic relationship for maintaining their lives over a long period of time. The EPS matrix is heterogeneously dynamic, for its composition and structure change over time and upon various environmental conditions. Nevertheless, it has been widely accepted that the main components of EPS are proteins, polysaccharides, DNA and humic substances. Among them, proteins and polysaccharides have been identified as the dominant EPS components (Frølund et al., 1996; Bura et al., 1998; Dignac et al., 1998; Durmaz and Sanin, 2001; Sponza 2002, 2003). In an attempt to elucidate the function of EPS in forming and maintaining a biofloc matrix, most studies on flocculation and biofilm focus on proteins and polysaccharides.

Our knowledge of the structure of EPS has grown in recent years. Two different EPS layers in a floc matrix have been proposed (Gehr and Henry, 1983; Jorand et al., 1995; Laspidou and Rittmann, 2002): (i) bound EPS refer to those tightly bound to cells that are relatively difficult to extract; (ii) soluble EPS are those loosely embedded in the floc matrix that are relatively easily to extract. This is consistent with the different layers proposed in bioflocs.

2.4.2 Function of EPS

There are two major roles of EPS in a floc matrix: as a bridge for holding bacterial cells together and as a sink for extracellular enzymes (Laspidou and Rittmann, 2002). Since EPS have many negatively charged functional groups, such as amino acid groups of proteins, they can connect with positive divalent ions, such as Ca^{2+} and Mg^{2+} in the surrounding media, to form a bridge holding cells together. In addition, EPS provides a microenvironment for accommodating various extracellular enzymes. In general, these enzymes are excreted from cells, to depolymerize large organic particles into small parts, serving as a food source for the cells. Therefore, EPS are important in developing and regulating a complex system that integrates various microbial communities.

Early studies on the function of EPS in holding microbial cells focused on the amount of EPS rather than the composition of EPS. Pavoni et al (1972) studied the EPS extracted from municipal activated sludge, and found an increase in EPS amount per microorganism occurred during microbial aggregation. There was a positive correlation between cell aggregation and the amount of EPS. In addition, adding EPS into an inorganic suspension (kaolinite, silica or alumina) induced flocculation of the system. All these results demonstrate the importance of EPS in the microbial aggregation.

2.4.3 EPS in Myxobacteria

With considerable efforts made in identifying the EPS components, researchers have paid much attention on the importance of EPS composition in microbial aggregation. To date, it is generally accepted that it is the EPS composition rather than the total amount that play a key role in microbial aggregation (Frølund et al., 1996; Higgins and Novak, 1997; Bura et al., 1998; Dianac et al., 1998; Durmaz and Sanin, 2001; Liao et al., 2001; Sponza 2003). The EPS components characterize the structures, surface properties and function of EPS. Thus, the essential role of EPS proteins and polysaccharides in microbial aggregation has been emphasized, as will be further discussed in the following sections.

Due to the complexity of aggregation of mixed species, many studies focus on single species as a starting point to elucidate the functional role of EPS in cell aggregation. In this regard, EPS in myxobacteria have been well studied. Myxobacteria are gram-negative soil prokaryotes that have characteristics of gliding motility and forming fruiting structures (Behmlander and Dworkin, 1994b). *Myxococcus xanthus* cells have been shown to be able to maintain a constant high-density population and exchange extracellular signals in regulating their life cycle. When the high-density cells experience starvation, the cells will glide as groups (social motility) and form a fruiting body like a biofilm. Two types of gliding motility are present in *M. xanthus*: adventurous (A) and social (S) motility. “A” motility is required for individual cell moving whereas “S” motility is needed for group gliding (Shimkets, 1986; Arnold and Shimkets, 1988;Weimer et al., 1998). Evidence has shown that the “S” motility of *M. xanthus* requires the aid of special appendages.

Two types of extracellular appendages, i.e., pili (fimbriae) and fibrils, have been identified in *M. Xanthus* (Arnold and Shimkets, 1988;Weimer et al., 1998). Pili are cell-length long (ca. 10μm) and 8 nm in diameter. They may draw cells closer through a side-by-side contact. Fibrils are bigger and longer than pili, usually as long as 10 cells and 30-50 nm in diameter. Fibrils attach onto the sides and ends of the cells and form a dense network. Both pili and fibrils are important for the social motility of *M. Xanthus*.

In particular, the structure and function of fibrils in mediating cell-cell interactions of *M. xanthus* has been explored (Behmlander and Dworkin, 1994a,b; Chang and Dworkin, 1994; Dworkin, 1999). The fibrils consist of a polysaccharide backbone with an approximately equal amount of adhering proteins. When a protease was added into the fibrils, to digest proteins in the matrix, it had little impact on fibril structure. On the contrary, when periodic acid HIO_4 was added to remove polysaccharides, the fibril structure was destroyed, suggesting that compared to proteins, polysaccharides have a more structural role in the fibril matrix (Behmlander and Dworkin, 1994a). To find out the function of fibrils in cell cohesion, Chang and Dworkin (1994) tested a group of social motility mutants *dsp*. Dsp is unable to produce fibrils, thereby failing to cohere and undergo a normal development. When extracellular fibrils were extracted from wild-type *M. xanthus* cells and added to *dsp*, the *dsp* cells exhibited an ability for aggregation and development. This demonstrates that fibrils are important in cell cohesion and development in *M. xanthus*.

Besides the role in cell-cell contact, fibrils participate in cell-cell signalling by offering a microenvironment for accommodating important enzymes and signal molecules (Kaiser and Losick, 1993; Behmlander and Dworkin, 1994a,b; Dworkin, 1996). Some external signals have

been found in regulating cell aggregation under stress conditions. For example, when myxobacteria cells are exposed to nutrient starvation, they tend to form a fruiting body: the starvation signal triggers the release of proteases. The proteases generate an "A" signal, which is a mixture of amino acids and peptides. The concentration of "A" signal is directly associated with the cell density. Once the cell density is sufficiently high, there is a sufficient concentration of "A" signal initiating the aggregation of cells to form a fruiting body.

Since proteins and polysaccharides are the dominant EPS components of both single and mixed species, elucidating their individual role in EPS should enhance the understanding of EPS in cell aggregation. The following sections will review studies on proteins and polysaccharides, respectively, especially on their function in cell aggregation with and without substratum.

2.5 EPS in Aggregation of Suspended Cells

2.5.1 Proteins in Cell Aggregation

The importance of proteins is highlighted in yeast flocculation (Calleja, 1987; Bony et al., 1997; Javadekar et al., 2000). A cell-cell interaction is formed by a connection between a cell-wall protein and a mannan site on the surface of neighbouring cells. FLO1 has been found to be one of the dominant genes in yeast flocculation. Bony et al. (1997) investigated the distribution and function of Flo1p protein in S*accharomyces cerevisiae* (strain ABXL-1D). They found that Flo1p was a cell wall protein. It was anchored to the membrane and then incorporated to a cell wall, making it available for cell-cell interaction. A specific region of Flo1p (N-terminal domain) has been shown similar properties as lectin and likely is involved in cellular aggregation. However, Flo1p is not the only glycoprotein involved in the cell cohesion.

Javadekar et al. (2000) analyzed another flocculent strain of S*accharomyces cerevisiae* (strain NCIM 3528). They found that a cell surface lectin had a similar amino acid residue to the Flo1p and played an important role in strain flocculation. This type of lectin was stable in acidic environments and was highly hydrophobic. Ferreira et al. (1994) and Moreira et al.(2000) also reported that p37 protein was involved in flocculation of yeast strains H*ansenula anomala* and K*luyveromyces marxianus*. If the genes encoding p37 protein were transferred to a non-flocculent strain, the transformed strain was able to flocculate. Therefore, it is not surprising that different flocculent yeast strains have different types of proteins involved in the flocculation.

In cell cohesion of M. x*anthus*, five major proteins have been identified to date as integral fibrillar proteins class 1 (IFP-1) (Behmlander and Dworkin, 1994b; Dworkin, 1999; Kearns et al., 2002). These five proteins have different molecular sizes (14 - 66 kDa) but the same amino acid composition. When fibrils were removed, the cells became unable to cohere, nor to glide nor to form fruiting bodies (Dworkin, 1999). Thus, researchers speculated that though the IFP-1 proteins might not be required for physical structure of fibrils, they were important in maintaining proper function (e.g., cohesion) of fibrils. Apart from IFP-1 proteins, Kearns et al (2002). identified a fibril protein A (FibA) in extracellular fibrils of *M. xanthus*. It shows that FibA did not have a significant effect on the structure and cohesion function of fibrils. However, the cells without FibA would produce elongated-shape fruiting bodies in comparison to round-shape of fruiting bodies in wild-type cells.

Compared to various studies on pure species, limited investigation is on elucidating the role of proteins in aggregation of mixed-species in suspension (e.g. activated sludge bioflocs). In

studying the effect of ionic strength on activated sludge, Higgins and Novak (1997) concluded that proteins were important in flocculation of bioflocs. With addition of divalent ions, bound protein concentrations were increased. Digestion of proteins with a pronase resulted in deflocculation of bioflocs characterized by increasing number of small flocs (5-40μm), whereas a polysaccharide-degrading enzyme (cellulase) caused no significant impacts on the bioflocs. The release of polysaccharides in the digestion of proteins indicated that proteins and polysaccharides were cross-linked in floc matrix. Using gel electrophoresis (sodium dodecyl sulphate polyacrylamide gel electrophoresis, i.e., SDS-PAGE), proteins functioning in flocculation were identified to be lectin-like, typically located on the appendages of bacterial cells (e.g., pili).

2.5.2 Polysaccharides in Cell Aggregation

EPS polysaccharides were identified much earlier than proteins. Actually, the name EPS had long been used as extracellular polysaccharides before the important role of proteins was accepted. Even in recent years, the term extracellular polysaccharides and EPS are still used interchangeably. In studies on cell aggregation of single- or mixed- cultures, some researchers stressed the importance of polysaccharides (Tago and Aida, 1977; Eriksson and Hardin, 1984; Horan and Eccles, 1986; Burdman et al., 2000).

In general, EPS polysaccharides are either homopolysaccharides with a repeating monosaccharide, or heteropolysaccharides composed of different monosaccharides. Sutherland (1994) described various types and properties of microbial exopolysaccharides, including curdlan, scleroglucan, xanthan, gellan and alginate. The author commented that the conformation of these polysaccharides was subject to change with temperatures, pH or ionic

strength. For instance, xanthan has a regular helical conformation. When temperature is increased, the double helix shifts from an ordered to a disordered from. In general, microbial polysaccharides are less stable and more easily degraded in the disordered form. Adding a cation (monovalent or divalent) could stabilize the conformation by increasing melting temperature of the helix.

An early study on identifying the difference of polysaccharides between floc-forming bacteria and suspended cells suggests that mucopolysaccharides are responsible for flocculation (Tago and Aida, 1977). By isolating a floc-forming bacterial strain from phenol-adapted activated sludge, Tago and Aida (1977) compared the polysaccharides extracted from the strain to those from suspended cells, and found a minor amount of mucopolysaccharides, composed of glucosamine, glucose, mannose, galactose and rhamnose. Though the amount of mucopolysaccharides was only 10% of total polysaccharides, mucopolysaccharides were speculated to account for the flocculation of bioflocs.

The alginate theory for formation of bioflocs emphasizes the positive role of alginate in flocculation (Bruus et al., 1992; Davies et al., 1993). Alginate is different from the other polysaccharides since it is composed solely of uronic acid residues, typically repeating L-guluronic (G) and D-mannuronic (M) acids. It is usually specific to Ca^{2+}, forming a firm gel. Since the bacteria producing alginate, such as *Pseudomonas aeruginosa, Azotobacter* sp., are found in activated sludge, alginate was considered to be associated with cation-inducible flocculation in the early flocculation research. But the fact that adding Mg^{2+} also improves sludge flocculation makes more researchers turn to other mechanisms instead of alginate theory.

It is evident that EPS polysaccharides are involved in the flocculation of N_2-fixing organisms as well. *Azospirillum* cells are free-living N_2-fixing organisms and tend to aggregate together in response to a high ratio of carbon to nitrogen. Burdman et al. (2000) investigated the aggregation ability of four *Azospirillum brasilense* strains by evaluating the composition and amount of extracellular polysaccharides. The different strains demonstrated different flocculation capacities along with various amounts and composition profiles of polysaccharides. Thus, the authors concluded that extracellular polysaccharides were involved in flocculation of *Azospirillum* species.

2.6 EPS in Biofilm

To investigate integration and disintegration of microbial communities, an attempt to understand the development of biofilm could be highly related and provide valuable insight for microbial flocculation and deflocculation.

Biofilms are defined as microbial communities attached on a solid surface. It has been recognized that a microbial community is not a simple accumulation of individual cells. Rather, single or various species of cells within the community are able to develop an interdependent relationship and often function as an integral unit with a high level of organization. This will enable the microorganisms to better survive in a dynamic environment (Davey and O'Toole, 2000; O'Toole et al., 2000). Water-filled channels inside the biofilm allow nutrients to come in and metabolic products to come out. Similar to an aggregated community in a liquid suspension, biofilms contain various bacterial cells surrounded by EPS. EPS are mainly responsible for the structure and physical properties of biofilms (Flemming et al., 2000). Biofilm development is a highly regulated process, involving various external and internal

signals, cell-cell communication and interspecies interactions. Despite the fact that mixed-species biofilms dominate in most environments, most current research focuses on single-species biofilms.

In discussing the development of biofilms, the role of quorum sensing is relevant. Quorum sensing is a way of bacterial intracellular communication that has been observed in facilitating biofilm production and integrity (Decho, 2000;von Bodman et al. 2003; Waters and Bassler, 2005). Not only within the same species, quorum sensing is also present in communication of interspecies. It has been shown that cell-to-cell communication is necessary for establishing a highly regulated architecture of biofilm. Bacteria communicate and coordinate through density-dependent signalling molecules called autoinducers. Quorum sensing is involved in biofilm initial formation, biofilm development, and even detachment of biofilm. Stoodley et al. (2002) surmised that in the presence of a relatively high level of cell density, quorum sensing could play a role in triggering the release of EPS-degrading enzymes, giving rise to the detachment of cells from biofilms. Puskas et al. (1997) reported that a quorum sensing system has been found in a free-living photoheterotrophic bacterium *Rhodobacter sphaeroides*, to prevent production of extracellular polysaccharides, and in turn, to prevent cellular aggregation. Based on the study on *R. sphaeroides*, Greenberg (2003) proposed that quorum sensing was involved in the detachment of biofilms.

2.6.1 Proteins in Biofilm

The indispensable role of EPS proteins in biofilm formation is recognized from initial attachment to an abiotic surface, through to biofilm development and biofilm detachment. Following are a few examples of proteins in biofilms of single- and mixed-species.

Using *Pseudomonas fluorescens* WCS365, O'Toole and Kolter (1998) investigated the involvement of proteins in the initial attachment to an abiotic surface. The authors found that protein synthesis was required for the early attachment, to maintain the cell adhesion on the abiotic surface. The addition of a protease (Pronase E) decreased the amount of attached cells but without causing cell lyses. This implied that one or more extracellular proteins were involved in the early interaction with the abiotic surface. Further analysis of mutants of *P. fluorescens* defective in the initial attachment supported the idea of participation of specific proteins in the initial biofilm formation.

An outer membrane protein, Antigen 43 (Ag43), appears to be involved in cell-to-cell and cell-to-surface interaction of *E. coli* biofilm growing in glucose-minimal medium (Danese et al., 2000b). The authors reported that the cells deficient in Ag43 were often unable to form biofilms. Therefore, it was speculated that Ag43 was involved in developing a *E. coli* biofilm.

Sauer et al. (2002) characterized four major stages of biofilm development in *Pseudomonas aeruginosa* and a distinct protein pattern was found at each stage. These four stages are reversible attachment, irreversible attachment due to production of EPS, biofilm development and detachment. Biofilm development is a complex but highly regulated process, involving different proteins in various regulatory pathways. For example, at the stage of development, Arc proteins, related to anaerobic processes, were up-regulated, indicating that oxygen became limited in some zones. In the final stage of detachment, the protein patterns became close to those in planktonic bacteria.

In the stage of biofilm detachment, the mechanisms remain unclear as to why microorganisms tend to detach from biofilms and what is the precise pathway for triggering and

regulating the detachment. It is speculated that some environmental factors (e.g., nutrient starvation) could trigger the production or release of matrix-degrading enzymes, imposing the detachment of cells from biofilm (Boyd and Chakrabarty, 1994; Lee et al., 1996; Allison et al., 1998; O'Toole et al., 2000; Stoodley et al., 2002). Lee et al. (1996) measured the activity of an endogenous surface protein-releasing enzyme (SPRE) in biofilm of *Streptococcus*, and found that detachment of the biofilm was mediated by the SPRE.

Proteins have also been shown to play a special role in plaque formation on teeth (Kolenbrander and co-workers, 1985, 1993; Ofek et al., 2003). Plaque is a biofilm composed of hundreds of various bacterial species. A coating of glycoproteins and mucins first cover a clean teeth surface, serving as a basis for adhesion of primary microorganisms. After the organisms bind to the proteins, cell-to-cell interactions and co-aggregation occur among the same and/or different species. Many proteins, such as lectin-like proteins and lipoproteins, again are involved in this step, aiding in forming a tight biofilm on teeth (Kolenbrander and co-workers 1985, 1993).

Although there is a large body of information on proteins in diverse regulatory circuits, it should be noted that the current understanding of proteins, with respect to structural and functional roles, is still far from a complete picture. The above discussion on proteins is limited to its important function in cell aggregation, cell-to-cell cohesion and cell-to-surface adhesion. Alternatively, the role of proteins in stress responses will be discussed in Section 2.7.

2.6.2 Polysaccharides in Biofilm

The involvement of polysaccharides in biofilm development has been described in different studies on biofilm of single-species and there is much debate on their functional role.

McKenney et al. (1998) reported that capsular polysaccharide/adhesin was able to mediate the initial attachment of gram-positive *Staphylococcus epidermidis*. In contrast, Danese et al.(2000a) used *E. coli* K-12 to evaluate the potential role of exopolysaccharides in biofilm development by tracking colanic acid, an extracellular polysaccharide produced by *E. coli*. They found that both strains with and without colanic acid production had an ability to attach on an abiotic surface. Thus, colanic acid production was not required for the initial cell attachment to a surface. Rather, it might be involved in forming a complex three-dimensional biofilm structure. Using *vibrio cholerae* E1 strain (gram-negative), Watnick and Kolter (1999) presented a similar notion that extracellular polysaccharides were involved in developing a three-dimensional biofilm. Collectively, different EPS polysaccharides may possess different functions and it is not feasible to generalize the role of polysaccharides in biofilm formation.

Besides involving in biofilm development, polysaccharides play a protective role in biofilms as well. EPS polysaccharides are not only an integral structural part of biofilm, but also act as a protection for anti-microbial agents. Of particular interest, polysaccharides were reported to protect microbial cells from extraneous environmental stresses, such as UV radiation, pH changes, toxic shock and osmotic stress (Davey and O'Toole, 2000).

Currently, there is still much debate on structure role or functional role of polysaccharides in cell aggregation. Some researchers reported that polysaccharides were more a structural component (Behmlander and Dworkin, 1994a; Flemming and Wingender, 2001; Ahimou et al., 2007). Others suggested that they were a functional component (Tago and Aida, 1977; Burdman et al., 2000). Regardless of controversy of these findings, it is clear that different

species, various environmental conditions, experimental strategies and heterogeneity of bioflocs affect the observations.

On the basis of above discussion, there is no doubt about the important role of EPS in the formation of microbial bioflocs or biofilms. Though understanding the precise role of EPS responding to environmental disturbances is not complete, it should be particularly noted the functional role of proteins in holding bioflocs together.

In addition, to formulate the mechanisms for disintegration of stressed cells, an in-depth understanding of cell stress responses is required. Thus, the following part of this review focuses on stress response of uni- and multi-cellular organisms.

2.7 Microbial Stress Responses

Despite the fact that a relatively large body of knowledge is available on responses of pure microbial species to environmental stresses, understanding responses of consortia of various microbial cells to environmental disturbances is limited. A biofloc is essentially a community accommodating different microorganisms living interdependently. Strategies for such a community to cope with the disturbances may not be a simple accumulation of the responses of individual cells. Nevertheless, to fundamentally understand the responses of bioflocs to environmental stresses, reviewing the stress responses of pure species is a logical step to start.

Considerable work has been conducted on microbial responses to various adverse stresses. This review highlights important features of some specific stresses, including heat and cold-shock, osmotic stress and oxygen variations.

2.7.1 Heat Shock

A group of proteins, called heat shock proteins (HSPs) are induced when bacteria are exposed to a heat shock. Early work on the HSPs started from *E. coli*, providing a basic understanding of regulation and function of HSPs. HSPs are a key factor in maintaining the regular function of proteins under both normal and stressed conditions. Some of the HSPs help repair misfolding or damaged proteins due to a high temperature, the others are required for cell growth under a high temperature (Yura et al., 2000). In *E. coli*, the induction of HSPs is regulated by a σ^{32} factor, a transcription initiation factor expressed under a heat stress. The level of σ^{32} is low at a normal temperature (30°C), and can rapidly increase upon a shift to 42°C within 5 min. Currently, around 30 HSPs (from 16 to 89 kDa) regulated by σ^{32} have been identified and the function of some HSPs still remains unknown. Yura et al. (2000) provides a detailed discussion on regulatory mechanisms of heat shock responses in *E. coli*. In eukaryotes, the regulatory pathways of HSPs may be more complicated, but essential function is similar to prokaryotes (Voellmy, 1996).

2.7.2 Cold Shock

Similarly, a group of cold shock proteins are induced in *E. coli* upon temperature downshifts. In contrast to the heat shock response widely studied from prokaryotes to eukaryotes, the cold shock response has received limited attention. Phadtare et al. (2000) conducted a study on *E. coli* and B*acillus subtilis* responding to low temperatures, providing a preliminary insight into adaptive responses of cold shock. CspA proteins were found in both *E. coli* and *B. subtilis*. They were present as a low level at 37°C but dramatically increased to a high level after a downshift to 15°C. The CspA proteins were able to facilitate RNA translation

at low temperatures. Unlike heat shock proteins, no σ regulators have been found to date in cold shock responses. Thus, what triggers the activation of cold shock proteins remains unknown.

2.7.3 Osmotic Stress

For those living in the environments of moderate salinity, microorganisms use osmolytes, so-called compatible solutes, to adapt to an osmotic stress (Bremer and Kramer, 2000). Alternations in external salinity are common in nature and microorganisms have to adjust to the changes. The compatible solutes are produced through intracellular synthesis or up-taken from the environment, and are highly congruous with cellular function. The stressed cells accumulate a high concentration of the compatible solutes without interfering with cytoplasmic water molecules, to balance turgor pressure under osmotic conditions. Glycine betaine, proline, and trehalose are the common compatible solutes used by microbes. When the osmotic stress is removed, the cells are able to release compatible solutes, to mediate the turgidity according to the changing environment. Currently, it is still not well understood about the signalling pathway leading to synthesis of compatible solutes.

Synthesis of compatible solutes is not unique in responding to an osmotic stress. Glycine betaine, as a compatible solute in *Listeria monocytogenes* (a gram-positive food-borne pathogen), has been found to improve cryotolerant ability of the organisms as well (Ko et al., 1994). Similarly, for *Bacillus subtilis*, glycine betaine and proline can function as a thermo-protectants to allow the cells growing under 52°C (Holtmann and Bremer, 2004).

2.7.4 Oxidative Stress

Reactive oxygen species, such as superoxide and hydrogen peroxide, can cause damage to cellular compounds by reacting with some lipids, proteins and DNA. The free radicals in superoxide anion (O_2^-) could interact with polyunsaturated fatty acids in the cell membrane lipids, and change the structure, permeability, flexibility and fluidity of the membrane. The free radicals can affect protein activity, such as ionic channels and ATPase activity, leading to a negative consequence (Haddad and Jiang, 1993).

Specific proteins are activated to improve the adaptive ability of cells to an oxidative stress. Approximately 40 different proteins are induced in *E. coli* cells responding to a H_2O_2 stress. Among them, OxyR protein was first discovered. Storz and Zheng (2000) presented a detailed description about the understanding of OxyR, including its regulation of expression of different genes, the chemistry of sensing H_2O_2, and its presence in other bacteria. Interestingly, the OxyR protein was also found to be a repressor of agn43 gene in *E. coli*, which encodes Ag43 protein for biofilm formation. However, the precise pathway of this repression is not clear.

2.7.5 Oxygen Limitation

Research has been dedicated to microbial responses to oxygen deprivation at a molecular level. Of particular interest, responses of mammalian neuronal cells oxygen deficits have long been a popular research topic since a large number of adults and immature infants suffer related anoxic injury. Following is the discussion on multicellular and unicellular organisms responding to a low oxygen concentration.

2.7.5.1 Responses of Mammalian Cells to Hypoxia

Responses of mammalian cells to hypoxia have been extensively studied. Hypoxia refers to an oxygen concentration below normal levels in air, blood or tissue. Many physiological responses are reported, such as increasing production of red blood cells and facilitating ventilation through the carotid body (Safran and Kaelin, 2003).

In general, hypoxia effect is divided into two categories according to its time scale: acute effect and chronic effect. The acute effect is often within seconds or minutes, while the chronic effect can last for hours and even days (Haddad and Jiang, 1993). The acute damage involves chemical modification of proteins after their translation to alter the activity of the proteins. As a comparison, long-term damage often induces gene expression for new proteins or improves the synthesis of existing proteins, to cope with the stress conditions. Cell responses to hypoxia could vary from adaptation, injury, to cell death depending on the extent of oxygen availability.

Although most protein syntheses are depressed under oxygen deprivation, some of them are up-regulated. A group of factors, identified as hypoxia-inducible factor (HIF), is reported to play an important role in activating genes for both adaptive acute and chronic responses (Kaelin, 2002; Safran and Kaelin, 2003). HIF consists of one α subunit. The presence of an oxygen degradation domain makes α unit unstable under a normal oxygen level. A considerable increase in the amount of HIF-α subunits occurs under the hypoxic condition. The molecular basis for HIF-α was delineated by Lando et al. (2003) and Semenza (2001).

Many studies have focused on oxygen sensing as the first step in understanding the responses to hypoxia. In order to adapt to hypoxic conditions, cells have to be able to detect the oxygen concentration first, followed by initiating signals to activate specific responding

pathway. The precise mechanisms by which multicellular organisms sense oxygen remain undetermined, despite some hypothesized models were proposed (Ratcliffe et al. 1998; Semenza 1999; De Marco and Caniggia, 2002).

Haddad and Jiang (1993) summarized hypoxic effects (acute and chronic) on mammalian neuronal cells. It is clearly shown that the whole response system is highly complicated and interacted. One of the direct responses to oxygen deprivation is declining ATP concentration, which imposes a series of changes in membrane potential, ionic transporters (ion channels and ion pumps), nutrient uptake, and cell injury. Following is a brief discussion of hypoxic effects on ionic fluxes across the cell membrane.

2.7.5.1.1 Ionic Fluxes Under Hypoxia

Membrane-bound ion channels and ion pumps are importance in transporting ions in and out of cells for their regular metabolism. Ion channels and ion pumps are membrane-spanning proteins and are highly specific to types of ions. Different from ion channels, ion pumps allow ions to move against a concentration gradient or electrical potential.

Apart from ion channels and ion pumps, ionophores are able to facilitate the transport of specific ions across the cell membrane. They are organic compounds smaller than membrane proteins. An example of an ionophore is valinomycin, an antibiotic for actively transporting K^+ across the cell membrane (Dobler, 1981). Valinomycin is a ring-shaped polymer capturing K^+ ion in the centre and hydrophobic tales towards outside. Such a structure allows it to penetrate the hydrophobic lipid bilayer and transport K^+ based on electrochemical gradients (Alberts, et al., 1983).

Hypoxic impacts on changes in membrane-bound transporters of Na^+ and Ca^{2+} ions have been demonstrated in mammalian cells (Haddad and Jiang, 1993). In general, a Na^+-K^+ ATP-dependent pump actively pumps Na^+ out of the cell and pumps K^+ into the cell, to maintain the gradients of Na^+ and K^+ across the cell. Transport of nutrients, such as amino acids and glucose, as well as other ionic species (H^+, Ca^{2+}) are mediated by these gradients. The function of such a pump relies on the supply of intracellular ATP. When the cells are exposed to oxygen shortage, intracellular ATP will decrease and activity of the Na^+-K^+ ATP-dependent pump will be inhibited, thereby resulting in a disturbance to the gradients of Na^+ and K^+. More Na^+ will remain inside the cells. Due to the accumulation of intracellular Na^+, the cells become unable to pump Ca^{2+} out of the cells via a Na^+-Ca^{2+} exchanger. Thus, extra Ca^{2+} can be retained inside the cells as well. The hypoxic condition can also trigger the release of Ca^{2+} from intracellular stores into cytoplasm. All of these will give rise to an accumulation of Ca^{2+} inside the cells, which is toxic to cells and often leads to cell injury (Haddad and Jiang, 1993; Jiang and Haddad, 1994).

Levels of ATP affect the activity of specific K^+ channels as well (Haddad and Jiang, 1993; Jiang and Haddad, 1994; Yamada et al., 2001; Barneo et al., 2004). Using ion-selective microelectrodes, Haddad and Jiang (1993) demonstrated a reduction in intracellular K^+ and an increase in extracellular K^+ under a hypoxic condition. The authors proposed that a decrease in the ATP concentration under oxygen deficiency induced an opening of ATP-dependent K^+ efflux channels, causing a leakage of K^+ into extracellular surroundings.

Besides ATP levels, other cytosolic factors, such as cellular Ca^{2+}, are involved in modulating the activity of K^+ efflux channels (Jiang and Haddad, 1994). There is increasing

evidence that changes in intracellular Ca^{2+} will activate specific Ca^{2+}-dependent K^+ efflux channels. Marrion and Tabalin (1998) demonstrate that in hippocampal neurons of adult rats, the influx of Ca^{2+} via a voltage-gated Ca^{2+} channel will trigger an opening of a Ca^{2+}-dependent K^+ efflux channel, though the molecular mechanisms are still unclear. Not only in animal cells, Ca^{2+}-dependent K^+ efflux channels have been found in plant cells (Bauer et al., 1998).

In preventing specific ion fluxes across the membrane, various chemicals for blocking ion channels, especially K^+ channels, have been investigated in eukaryotic species, including tetraethylammonium (TEA), charybodotoxin (ChTX), iberiotoxin (iBTX), glibenclamide, etc. TEA is the most widely used non-selective blocker and often is the first choice for probing the structures and functional properties of K^+ channels. Langton et al. (1991) reported the use of TEA in effectively blocking Ca^{2+}-dependent K^+ channels in mammalian muscle cells. Glibenclamide is another K^+ channel blocker but mainly targets on ATP-sensitive K^+ channels (Haddad and Jiang, 1993; Kažić and Gojković-Bukarica, 1999). By adding glibenclamide, Haddad and Jiang (1993) successfully observed a significant attenuation in the increase of extracellular K^+ under hypoxic conditions.

2.7.5.2 Responses of Unicellular Organisms to Hypoxia

Knowledge on how bacteria cope with the stress of excessive oxygen or oxygen starvation is accumulating. Intracellular oxygen concentration usually is maintained within a narrow range, to maximize oxygen usage while minimizing the impact of excessive oxygen. Many survival strategies under oxygen variations have been exploited, including using an alternative electron acceptor, down-regulating genes for oxygen metabolism, and up-regulate genes for reduction of alternative oxidants (Potter et al., 2000).

Bauer et al. (1999) and Patschkowski et al. (2000) reviewed various regulatory circuits of *E. coli* to adapt to oxygen variations, depending on the extent and duration of oxygen stress. Oxidative stress induces OxyR or SoxR-SoxS regulatory mechanisms, to regulate the synthesis of new proteins to degrade hydrogen peroxide or superoxide. In response to oxygen limitation, *E. coli* could switch off genes for TCA cycle (citric acid cycle) through ArcB-ArcA regulator (arc represents aerobic respiration control) and switch on genes for anaerobic electron transfer through FNR proteins (fumarate and nitrate reduction). ArcAB and FNR systems function independently and co-operatively in regulating the metabolic pathways of *E. coli* responding to oxygen stress.

Ion transport systems have been identified in unicellular organisms, though their functional properties remain largely unknown. Transport systems of Na^+, K^+, NH_4^+ and Ca^{2+} in prokaryotes were reviewed by Bakker (1993) and Rosen and Silver (1987). Ion transport systems in *E. coli* have been studied in the most detail to date. In regard to K^+ transport, K^+ influx systems are different from K^+ efflux systems, providing *E. coli* cells with flexibility in response to various environmental disturbances. Several ATP-driven K^+ uptake systems have been demonstrated, including Kdp, TrkG and Kup. For K^+ efflux systems, besides glutathione-related K^+ efflux (KefC), K^+/NH_4^+ antiporter, K^+/H^+ antiporter and stretch-activated K^+ efflux channels (responding to mechanical forces from local stretching or compression) have been reported (Bakker, 1993).

Among the K^+ efflux systems in *E. coli*, KefC is best understood to date (Booth et al., 1993). The KefC system plays a role in regulating both turgor and intracellular pH in *E. coli*. It is suggested that cytoplasmic acidification via K^+ efflux confers the cells a protective strategy

against electrophilic toxin disturbances (Ferguson et al., 1995; Ferguson et al., 1997). The KefC system has been found in other gram-negative bacteria, but is rarely present in the gram-positive species (Douglas, et al., 1991).

By adapting the knowledge of KefC K^+ efflux system in *E. coli*, Bott and Love (2002) proposed that a similar transport system was present in activated sludge systems and played a key role in triggering deflocculation in response to a shockloading of toxic electrophilic chemicals. As described in Section 2.2.4, the authors suggested that the disturbance of electrophilic toxins could initiate a K^+ efflux through the KefC channel, actively releasing cellular K^+ into the extracellular environment. As a consequence, ionic strength of EPS is reduced, thereby causing deflocculation.

With respect to the function and structure, prokaryotic ion channels to some extent are similar to eukaryotic ion channels. Recently, MacKinnon and co-investigators probed the structure of prokaryotic K^+ channels (MacKinnon et al., 1998; Ruta et al., 2003; MacKinnon, 2004). They found a K^+ channel from *Streptomyces lividans* was structurally similar to eukaryotic K^+ channels. From an oceanic thermal vent, a voltage-dependent archaebacterial K^+ channel was structurally and functionally similar to eukaryotic voltage-dependent K^+ channels. This suggests that, despite the difference between prokaryotes and eukaryotes, studies on responses of eukaryotic ion channels to disturbances should provide a valuable insight on understanding stress responses of prokaryotic ion channels.

Besides K^+ transport, Ca^{2+} transport in bacteria has been reported, including *E. coli* and *Bacillus* genus (Rosen and McClees, 1974; Lynn and Rosen, 1987). Nearly every type of cell maintains a relatively lower cytosolic Ca^{2+} level than that in extracellular solution though the

reasons remain unclear. Thus, Ca^{2+} efflux system is the most important means to export Ca^{2+} out of cells. There are two major transport systems: ATP-dependent Ca^{2+} pump and antiporters, such as Ca^{2+}/H^{+} and Ca^{2+}/Na^{+}. For a detailed description of Ca^{2+} transport systems in different prokaryotic species, readers can refer to Lynn and Rosen (1987).

Ion channels have also been discovered in unicellular eukaryotic species, including paramecium, dictyostelium and yeasts (Martinac et al., 1994). Paramecium cells possess different channels for transporting Ca^{2+}, K^{+}, Na^{+} and Mg^{2+}, regulated by changes in membrane potential or intracellular Ca^{2+} levels. In yeast cells *S. cerevisiae*, a voltage-sensitive K^{+} channel has been found. The activity of this K^{+} channel is effectively blocked by TEA chloride. However, small sizes, vast diversity and unique structures (e.g., the presence of cell wall and/or outer membrane) make the studies on ion transport systems in prokaryotic and lower-eukaryotic organisms challenging.

Based on the aforementioned cation movements in mammalian cells responding to hypoxic conditions and the presence of ion transport systems in both prokaryotes and eukaryotes, it is plausible to surmise that similar ionic fluxes could occur in mixed consortia of various microorganisms (e.g., activated sludge), as a response to oxygen deprivation: The oxygen limit triggers the function of specific ionic transport systems, thereby releasing monovalent ions (e.g., K^{+}) into the extracellular surrounding and importing divalent ions (e.g., Ca^{2+}) into the stressed cells.

Besides the efflux of ions discussed above, the efflux of organic substrates is a common phenomenon in pathogenic bacteria. Recent studies on bacterial resistance to tetracycline have shown that an active efflux of tetracycline is one of the important means for the cells to survive

under the toxins (e.g., antibiotics) (Yamaguchi et al., 1990a,b, 1991; Schnappinger and Hillen, 1996; Nelson, 2002). Tetracycline is one of the common antibiotics used for bacterial infection. However, bacterial resistance to tetracycline has been found in both gram-negative and gram-positive species. Efflux of tetracycline in *E. coli* was first discovered in 1978 (Levy and McMurry, 1978) and was proposed to be one of the mechanisms for antibiotic resistance. Efflux of tetracycline in *E. coli* is mediated by an antiporter in which a tetracycline-divalent complex is extruded by a proton influx (Yamaguchi et al., 1990a,b). Not only in *E. coli*, an active efflux of tetracycline has also been found in *P. aeruginosa* (Li et al., 1994). Therefore, membrane bound efflux proteins appear to be a general strategy for bacteria to cope with disturbances.

2.8 Summary of Literature Review

Biological wastewater treatment plants are often exposed to various disturbances, such as fluctuations in substrate loading and DO concentrations, variations in temperatures, pH and toxicant control, etc. As one of the common upset consequences, disintegration of bioflocs under transients is not well understood.

Through this review, the important role of EPS proteins in dissociation of activated sludge bioflocs has been emphasized. Proteins are often the first inducible component under the stress. Thus, examining changes in extracellular proteins, including types and concentrations, is necessary for elucidating the underlying mechanisms of deflocculation in response to disturbances.

On the other hand, it is of interest to exploit the changes in extracellular ionic concentrations under disturbances, to elucidate the disintegration of bioflocs from a standpoint

of cation fluxes. Concentrations of cations, especially divalent and monovalent cations play a key role in controlling the aggregation of bioflocs. Given a large body of information available on stress responses and the knowledge on microbial ion transport systems, it is feasible to clarify whether the mixed consortia of various microorganisms in wastewater systems (i.e., activated sludge bioflocs) also have cation movements across the cell membrane in response to the disturbances (e.g., short-term low DO).

2.9 Significance of this Research

The above review on cell aggregation and responses to environmental stresses highlights the importance of EPS proteins in cell aggregation and stress responses, as well as the involvement of cation fluxes in mammalian cells responding to oxygen deficit. This forms the basis for experiments performed in this work on exploring the mechanisms for deflocculation of activated sludge. However, there is limitation on the current studies of activated sludge under disturbances, as summarized below:

- Currently, the fundamental understanding of deflocculation of activated sludge under a short-term low DO remains limited. A GGKE theory for deflocculation due to a shockloading of toxins was proposed by Love and Bott (2004). However, a question remains whether similar mechanisms account for deflocculation under different disturbances, or different mechanisms are for different stress responses.

- Changes in extracellular cations have not been examined in an activated sludge system exposed to DO disturbances. As aforementioned, specific cation fluxes through membrane-bound ion transporters have been proposed as a stress response of mammalian cells to O_2 limit. However, in an activated sludge system, profiles of

extracellular cations under the DO disturbances have not been studied. In exploring the fundamentals for deflocculation, it is worth examining the profiles of extracellular cations under the DO limitation.

- There is lack of a detailed examination of DO impacts on treatment performance in the current literature. So far, deflocculation and the impacts of short-term low DO have been investigated in batch systems or a continuous system fed with synthetic feeds, mainly characterized by the changes in turbidity and particle size distribution. In this research, in addition to batch tests, continuous experiments using industrial wastewater are performed. The DO impacts on treatment performance and properties of bioflocs are examined, with respect to changes in turbidity, SS, removal of SCOD, particle size distribution, EPS components, extracellular cations, and floc strength.

- Though the importance of proteins and polysaccharides in the aggregation of mixed species has been recognized, DO effects on the changes in EPS components have not been well identified in the current literature. This work examines the impacts of short-term low DO on EPS proteins, polysaccharides and humic substances in both batch and continuous systems;

- To date, no effective strategies for minimizing deflocculation are available. This study attempts to explore the solutions for deterring the deflocculation under short-term low DO by examining the effects of addition of Ca^{2+}, ion channel blockers and ionophores on DO-stressed samples.

In regard to the above limitation, this work attempts to fill the niche of identifying the effects of short-term low DO on activated sludge and understanding deflocculation under short-term low DO. To achieve this goal, different experiments in batch and continuous systems were carried out, which are delineated in Chapter 3.

CHAPTER 3 EXPERIMENTAL METHODOLOGY

3.1 Overall Approach

Through literature review, discussion with industrial personnel and a series of bottle tests (see Appendix A), a short-term low DO concentration was chosen to be the transient studied in this work. The DO transient is commonly present in wastewater treatment plants and is of industry concern. Studying the DO transient is relatively achievable and manageable at a laboratory scale. Deflocculation under the short-term low DO has received limited understanding. Ultimately, it is expected that the fundamentals for deflocculation are not DO-specific and are applicable to deflocculation under other disturbances.

The main experimental activities in this work were performed in a sequence of batch experiments on municipal activated sludge, continuous experiments using wastewater from a pulp and paper mill, and batch experiments on *E. coli* suspensions. A schematic diagram for the overall approaches and linkage of the experiments is depicted in Figure 3.1, followed by a detailed description of each experimental stage.

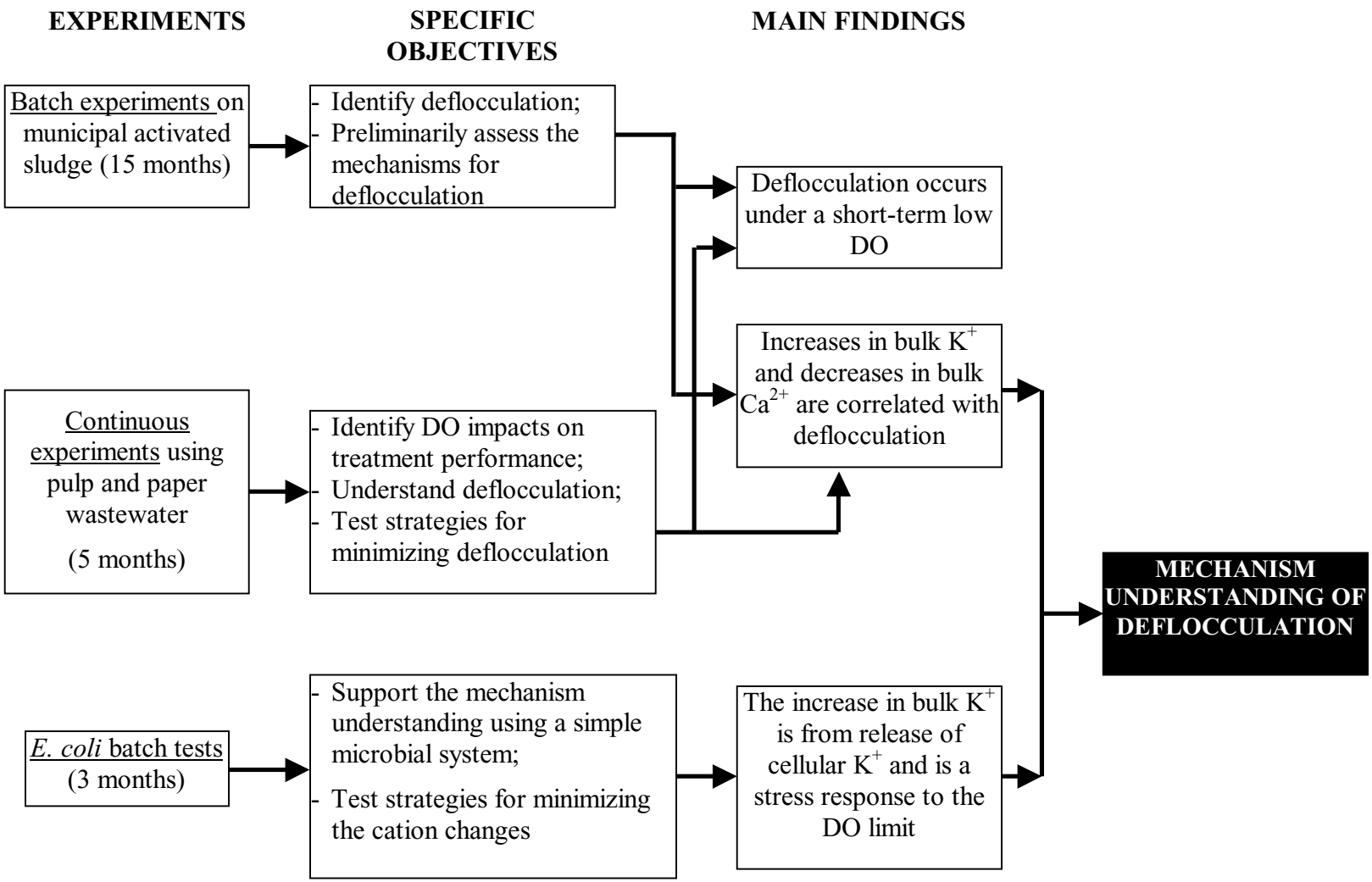


Figure 3.1 A summary of overall experimental approaches

3.2 Batch Experiments Using Municipal Activated Sludge

3.2.1 Experimental Strategies

A series of batch experiments on short-term DO transients were performed over a period of 15 months. A roadmap of the batch experiments conducted at different stages is summarized in Table 3.1. For each stage, at least one repeated experiment was conducted independently on different days, to achieve reproducible observations.

Table 3.1 A summary of batch experimental activities on municipal activated sludge

Subsection	Description	Main Parameters	Results
I	Identify deflocculation under short-term low DO	DO, pH, turbidity, SS, levels of EPS proteins, humic substances and polysaccharides, microscopic observations	Section 4.1.1
II	Distinguish pH effects from DO effects by adding phosphate buffer	Same as above	Appendix E
III	Perform enzymatic tests to identify the roles of proteins and polysaccharides	Turbidity, concentrations of extracellular proteins and polysaccharides	Section 4.1.2.2
IV	Examine changes in extracellular cations under the DO limit	Concentrations of extracellular K^+, Na^+, Ca^{2+} and Mg^{2+}, conductivity, surface charge, DO, turbidity, SS, EPS proteins	Section 4.1.1
V	Test the effects of external addition of K^+ and Ca^{2+}	Concentrations of extracellular K^+, Na^+, Ca^{2+} and Mg^{2+}, DO, turbidity, SS, bulk proteins	Section 4.1.2.1
VI	Analyze profile of proteins under the DO transients using gel electrophoresis (SDS-PAGE)	Protein concentrations, gel images	Yang (2005)

3.2.2 Experimental Setup

3.2.2.1 DO Experiments

Fresh mixed liquor of activated sludge was collected from the end of an aeration tank in North Toronto Treatment Plant and was transported to the laboratory within 45 min. Approximately 40,000 m^3/day wastewater is treated in the plant. The average sludge retention

time (SRT) is 6 days. The average temperature of mixed liquor varies from 14°C to 18°C over the seasons. The concentrations of mixed liquor suspended solids (MLSS) were 2100–3000mg/L during the experimental period.

Experiments on the DO transients were carried out in four 2-L parallel batch reactors (D x H = 8.5 cm x 47 cm) each placed on a magnetic stirring plate, as illustrated in Figure 3.2. The effective working volume was approximately 1.7 L. Two glass tubes inserted from the top of each reactor were to connect with a gas line and to hold a glass thermometer, respectively. The DO levels in the reactors were controlled by aeration from an air pump (Elite 800, Rolf C. Hagen Inc.) or by N_2 purging from a compressed N_2 cylinder (BOC Gases Com.). The stone air diffusers were positioned at approximately 0.4-L reactor volume from the bottom. Mixing in the reactors was provided by magnetic stirring bars (0.7x 0.7 x 5 cm^3) at the bottom. The operating temperature in each reactor was maintained at 25-26ºC through a circulating water jacket connect to a water bath coupled with an on-off thermal control.

Figure 3.2 A photograph of 4 parallel reactors

Approximately 1.7 litres of fresh mixed liquor activated sludge was loaded into each reactor immediately after an on-site collection, followed by the onset of air aeration and N_2 purging as time 0. One was a control reactor with air aeration at 160 mL/min, the other three were triplicate transient reactors with N_2 purging at 150-175 mL/min. The transient reactors were continuously purged with N_2 for first 6 hours. Within 5 min, DO levels in the transient reactors dropped below 0.5 mg/L, and were maintained at the low level for 6 hours. In the last 2 or 4 hours, re-aeration from air pumps (160 mL/min) was supplied to the transient reactors to restore the DO levels above 5 mg/L (Figure 3.3). In a typical batch experiment, the concentrations of MLSS in the reactors were approximately 2200-2500 mg/L throughout the experiments. The mixed liquor samples were taken every 2 hours and were analyzed immediately after the sampling.

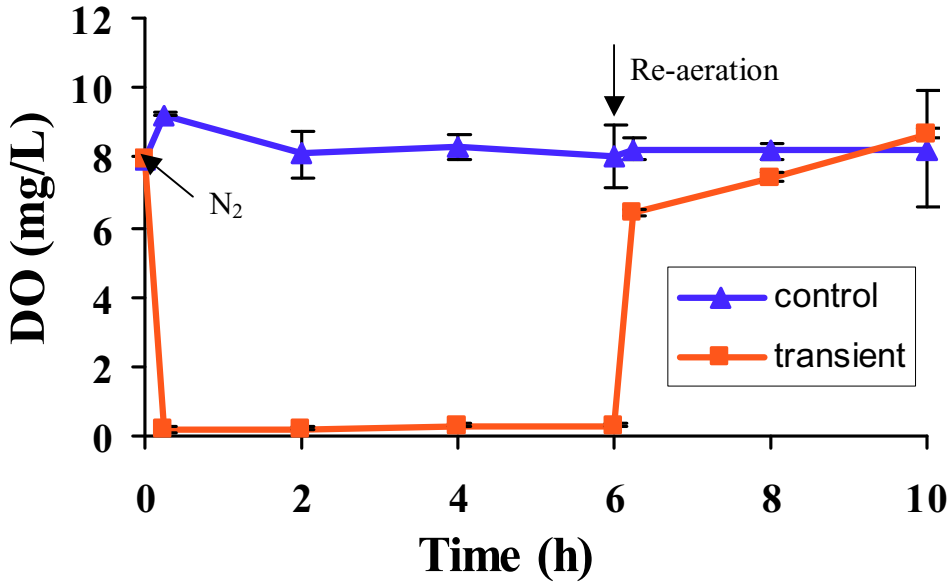


Figure 3.3 An example of DO profile in a typical batch experiment: DO level in the control reactor were kept around 8mg/L; DO levels in the transient reactors were below 0.5mg/L in the first 6 hours. The error bars are the +/- one standard deviation of DO measurements.

3.2.2.2 pH Control

In separate tests, phosphate buffer solution was used for maintaining pH around 7.5 under the DO limitation. It was observed that pH increased under N_2 purging, usually from 7.5 up to 8.5. Such an increase was attributed to a reduction in CO_2 production and continuous CO_2-stripping off the system. The pH changes may affect sludge surface properties, which, in turn, have an influence on the overall physicochemical properties of activated sludge. Therefore, to eliminate the pH effect on the system, 70 mL of phosphate buffer solution containing 0.38 M Na_3PO_4 (Sigma-Aldrich Com., 96%) and 0.35 M H_3PO_4 (Sigma-Aldrich Com., ≥85%) was added into one control and one transient reactors (Starkey and Karr, 1984). pH effect and potential influence from buffer solution would be distinguished from impacts of low DO.

3.2.2.3 Enzymatic Tests

Enzymatic tests were conducted to examine the important roles of proteins and polysaccharides in sludge flocculation. Sludge samples were treated with trypsin, cellulase, amylase, respectively, and were incubated for 15 min to 8 hours. Turbidity was measured at different times. 2 mL of Trypsin (Sigma-Aldrich Co. T-4549, 10x), a common proteolytic enzyme that degrades proteins was added to 18 mL of mixed liquor sample. 9 mg of cellulase (Sigma-Aldrich Com. C-1184) was added to 30 mL of mixed liquor sample for degradation of polysaccharides. Since municipal activated sludge from a starch-rich environment was used, amylase (Sigma-Aldrich Co. A-3176) was also attempted at a concentration of 1 mg of amylase in 30 mL of mixed liquor.

3.2.2.4 External Addition of K^+ and Ca^{2+}

To better clarify the relationship between sludge deflocculation and changes in bulk K^+ and Ca^{2+}, extra K^+ and Ca^{2+} were added into the system. 10 mL KCl (Sigma-Aldrich Com., ≥99%) solution at a concentration of 0.054 M was added into one control reactor (DO > 6mg/L) at time 0, to investigate whether the addition of extra K^+ can cause deflocculation of activated sludge. The amount of added K^+ was 1-fold higher than the increment of bulk K^+ observed under the DO limitation. Similarly, at time 0, 10 mL of $CaCl_2.2H_2O$ (Sigma-Aldrich Com., ≥99%) solution at a concentration of 0.1 M was added into one control reactor and one transient reactor, respectively. The amount of added Ca^{2+} was in a comparable quantity to the reduction in bulk Ca^{2+} observed under the DO limitation. It was expected to clarify whether adding Ca^{2+} would alleviate the reduction of bulk Ca^{2+}, thereby preventing deflocculation under the DO disturbance.

3.2.3 Sample Treatment and Analytical Techniques

Operating condition in each reactor was monitored by measuring DO levels, temperatures and pH. A DO probe (Oakton DO100, 35640-series) was used for the DO measurement. The membrane was replaced and the probe was cleaned regularly. Thermometer inserted in each reactor was to monitor the operating temperatures. A pH/conductivity probe (Oakton pH/CON10, 35630-series) was used for pH and conductivity readings.

The samples taken from each reactor were analyzed for turbidity, SS in supernatants, concentrations of biopolymers (i.e., proteins, polysaccharides and humic substances) and cations in EPS. A schematic diagram on sample treatment is presented in Figure 3.4.

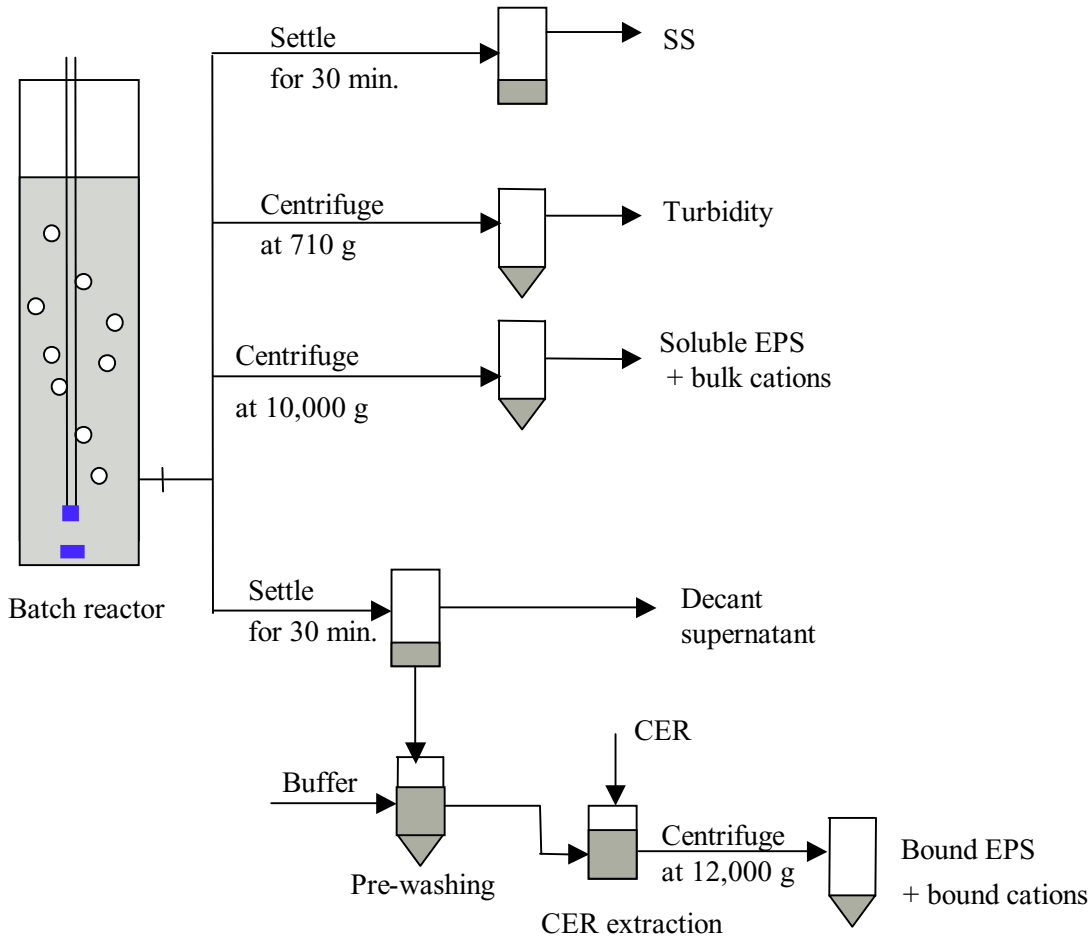


Figure 3.4 A schematic diagram of sample treatment in batch experiments

Turbidity: Supernatant turbidity was measured as absorbance at 650 nm after centrifugation (Allegra^{TM} 25R Centrifuge, Beckman Coulter Inc.) at 710 g (2000 rpm) for 2 minutes (Wilén et al., 2000a). To explore size range of the particles responsible for turbidity absorbance readings, series filtration using different membrane pore sizes was performed. It was found that mainly the particles sizing from 0.45 μm to 1.5 μm were counted in the absorbance readings.

MLSS and SS in supernatants: For MLSS measurement, 5 mL of mixed liquor was filtered by 1.5 μm filter paper (Whatman 934-AH) and solids on the filter paper were dried at 103-105ºC for 1 hour according to the standard methods for total suspended solid (TSS)

measurement (APHA et al., 1995, Section 2540D). For supernatant SS concentrations, 50 mL of mixed liquor sample was settled by gravity for 30 min, and the supernatant was taken for TSS measurement.

Soluble EPS: As discussed previously, soluble EPS are the portion that is extracted easily. 30 mL of mixed liquor sample was centrifuged at 10,000g and 4ºC for 15 minutes. The supernatant was regarded as a solution of "soluble" EPS (Higgins and Novak, 1997). The modified Lowry method (Hartree, 1972) was used for protein analyses with bovine serum albumen (BSA, Sigma-Aldrich Com., ≥96%) was used as a standard. In order to avoid the interference of humic substances in protein measurement, colorimetric absorbance for each sample was measured with and without addition of $CuSO_4$ (A_{total} and A_{blind} at 650 nm wavelength), respectively. Thus, the absorbance for protein concentration was corrected by the difference between the two readings (A_{total} and A_{blind}) (FrØlund et al., 1995). Detailed procedures for analyzing proteins and humic substances are presented in Appendix B. Polysaccharides were measured as glucose equivalent using the Anthrone method described by Raunkjaer (1994) and Liao (2000).

Bound EPS: The detailed procedures for extracting bound EPS are described by FrØlund et al. (1996) and Liao (2000). In brief, 110 mL of mixed liquor sample was extracted by the cation exchange resin (CER) method (at 1,000 rpm for 1 hour) (FrØlund et al., 1996). Then, the sample was centrifuged at 12,000 g and 4ºC for 15 min. The supernatant was taken for analyzing bound EPS components. Analyses of concentrations of proteins, humic substances and polysaccharides in the bound EPS are identical to the protocols described in the soluble EPS.

Extracellular cations: For measurement of cations (K^+, Na^+, Ca^{2+} and Mg^{2+}) in soluble or bulk liquid, 30 mL of mixed liquor sludge was centrifuged at 10,000 g and 4ºC for 15 minutes. The supernatant was filtered by 0.45 μm membrane syringe filters (Pall Corp.) before being analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). For sample storage, 1-2 drops of concentrated HNO_3 (VWR Com., 68-70%) were added into solution before the filtration and the samples were stored at 4ºC. For cations in bound EPS, after CER extraction and a high-speed centrifugation (12,000 g, 4ºC and 15 min), a portion of supernatant (> 5 mL) was filtered and analyzed by ICP-AES. It was assumed that cell lyses by centrifugation and CER extraction were minimal. Thus, bulk cations mainly refer to those extracellular cations that were dissolved in aqueous. In comparison, cations in bound EPS were mainly those floc-associated cations, i.e., were tightly encased in EPS matrix and were bound with EPS or cell surfaces.

Except K^+, Na^+, Ca^{2+} and Mg^{2+}, examining the levels of iron (Fe^{3+} and Fe^{2+}) and other cations was not considered at this stage. Past research carried out by Morgan-Sagastume (2003) showed a much lower level of Fe compared to the amounts of K^+, Na^+, Ca^{2+} and Mg^{2+}: the level of total Fe in pulp and paper wastewater was less than 0.5 mg/L whereas the levels of K^+, Na^+, Ca^{2+} and Mg^{2+} were 7-300 mg/L. In the mixed liquor activated sludge fed with the same industrial wastewater, the level of Fe was only 1-2 mg/g MLVSS. In the North Toronto Treatment Plant where the mixed liquor samples in this study were collected, the concentration of Fe in municipal incoming wastewater was monitored monthly during the sampling period and was shown be 0.2-1.4 mg/L. Thus, the levels of Fe were estimated to be low and only the four dominant species, K^+, Na^+, Ca^{2+} and Mg^{2+}, were considered in the cation measurements in this study.

A further examination was performed, with respect to identify whether the released soluble EPS under DO transients could re-associate with bulk Ca^{2+}. 1 mL of soluble EPS solution was acidified with 1 mL of 10% HNO_3 and heated under 100ºC for 30 min. The sample was then diluted by 10 mL of 2% HNO_3 (Bott and Love, 2002). By this treatment, the biopolymer-associated cations were released for analysis by ICP-AES.

3.3 Continuous Experiments Using Pulp and Paper Wastewater

3.3.1 Operating Strategies

Primary treated effluents from a bleached kraft pulp and paper mill were used in this study. Approximately 100,000 m^3/day wastewater flow into the treatment plant with 60% from a pulp mill (hardwood and softwood) and 40% from a paper mill. Secondary treatment plant in the mill is an aerated lagoon system, including two aerated cells in series and one quiescent zone. The total retention time of two cells is approximately 6 days. Average inlet COD within the collection period (April 2005) was 570 mg/L. 2,300 litres of wastewater was collected from the outlet of primary settling basins, together with 20 litres of mixed liquor from one of the aerated cells. The samples were transported to the laboratory under refrigerated conditions (4ºC) and then were refrozen at -20ºC. Since mixed liquor from the lagoon was of low concentration (< 200 mg/L), inoculum biomass for the experiments was a blending of one third of mixed liquor from the lagoon and two thirds of return activated sludge from a local municipal treatment plant (North Toronto Treatment Plant). 0.4 litre of biomass inoculum with a TSS concentration of 7940 ± 210 mg/L (TVSS = 5670 ± 150 mg/L) was introduced to each reactor and was aerated for 24 hours at room temperature before starting the experiments.

Over a period of 160 days, effects of short-term DO transients were examined in four parallel sequencing batch reactors (SBRs), a semi-continuous system including reactors (Figure 3.2) and auxiliary peristaltic pumps and pre-heating tanks. A schematic diagram of the SBR system is presented by Morgan-Sagastume (2003). Besides the basic apparatus setup described in Section 3.2.2.1, additional features are briefed herein: Operating of the whole system was controlled by on-and-off programmable timers (Indoor Digital Timer 7 Day Cycle, Mastercraft), including feeding, aeration, N_2 purging, mixing and effluent discharging. The top of each SBR was sealed by a stopper with 3 inserted glass tubes for feed, gas input (aeration or N_2 purging) and temperature measurement, respectively. Mixed liquor samples were taken at a 0.94-L outlet from the bottom of the reactor whereas effluents were discharged at a 0.68-L outlet from the bottom. The operating temperatures of SBRs were controlled at 27-29°C over the experimental period. Average mixed liquor concentration in each reactor was maintained around 1,500 to 2,000 mg/L throughout the experiments.

The SBRs were operated at an 8-hour cycle with 3 cycles per day. Each cycle consists of feeding, reaction, settling and discharge. Mixing and aeration/N_2 purging were started from feeding cycles and were stopped at the end of reaction cycles. The average sludge retention time (SRT) in four SBRs was maintained approximately 8 days by daily wasting an amount of mixed liquor near the end of reaction cycles (usually after 5 hours of aeration or N_2 purging). Hydraulic retention time (HRT) was approximately 14 hours with an effective working volume of 1.55 L in each reactor. The SBRs were divided into two control reactors (SBR1 and SBR2) and two transient reactors (SBR3 and SBR4), serving as duplicates for each condition. Aeration in each SBR was supplied by an air pump at a flow rate of 160 mL/min. N_2 purging in the transient cycles was supplied from a compressed N_2 cylinder and was maintained

approximately at 150–160 mL/min. A summary of operating conditions in SBRs is presented in Table 3.2.

Table 3.2 Operating conditions of SBRs

Parameters	Description
Effective reactor volume	1.55 litres
Cycle length	8 hours
Feeding	15 min.
Reaction	395 min.
Settling	60 min.
Discharge	10 min.
Temperature	27-29°C
pH	7.2-7.8 (Control) 7.3 – 8.6 (Transient)
DO	5.7-7.7 mg/L (Control) 0.1-0.4 mg/L (Transient)
SRT	8 days
HRT	14 hours
MLSS	1,500 – 2,000 mg/L

The feed to SBRs was prepared every other day and was stored in 4 separate 9-L containers (one per SBR) at 4°C in a refrigerator. To provide extra nutrients, nitrogen (N) and phosphorus (P) as NH_4Cl and $(NH_4)_2HPO_4$ were added to thawed, raw mill effluents in a SCOD:N:P ratio

of 200:5:1. One and a half hours before the feeding cycle, the feed from each container (approximately 0.9 L/SBR) was pumped into four separate 2-L glass pre-heating tanks (D x H = 9.5 x 34 cm) immerged in a water bath. The temperature of the feeds was slowly warmed up before being pumped in the reactors.

The history of operating condition in each SBR is illustrated in Figure 3.5, followed by DO profiles of the control and transient reactors throughout the experiments in Figure 3.6. Through the acclimation period (period I), the inoculum was able to adapt to the laboratory operating conditions and the SBRs reached a stable performance as shown by profiles in MLSS, turbidity, ESS, and removal of SCOD (see Appendix C). From days 42 to 90 (period II), deflocculation was demonstrated in the transient reactors. The DO levels in the control reactors SBR1 and SBR2 were maintained above 5 mg/L throughout the period. The transient reactors SBR3 and SBR4 underwent a periodic N_2 purging for 6 hours, followed by a recovery period of 6 days with sufficient aeration. However, one effluent pump was broken at the night of day 50 and caused overflow in control reactor SBR1 and a partial washout of biomass. Thus, another re-acclimation period was performed to stabilize the system till day 69. The DO transients were then resumed on day 70.

Strategies for deterring deflocculation under the short-term low DO were tested from days 91 to 153 (period III). From days 91 to 112, calcium chloride dihydrate was added into the daily feeds of one control reactor (SBR1) and one transient (SBR3) reactor. The amount of $CaCl_2.2H_2O$ was started with 40 mg/L feed and was increased up to 62.5 mg/L feed within 3 days. Before switching to the addition of another chemical, no addition of $CaCl_2.2H_2O$ was added into the daily feeds from days 113 to 115. From days 116 to 130, TEA chloride

($(C_2H_5)_4NCl$, Sigma-Aldrich Com., ≥99%) was added into the daily feeds to SBR1 and SBR3 starting from 0.17 g/L feed till 1.7 g/L feed within 14 days. Similarly, from days 136 to 151, glibenclamide ($C_{23}H_{28}ClN_3O_5S$, Sigma-Aldrich Com., ≥99%) was added into the feeds of SBR1 and SBR3 at a concentration of 5-33 mg/L feed.

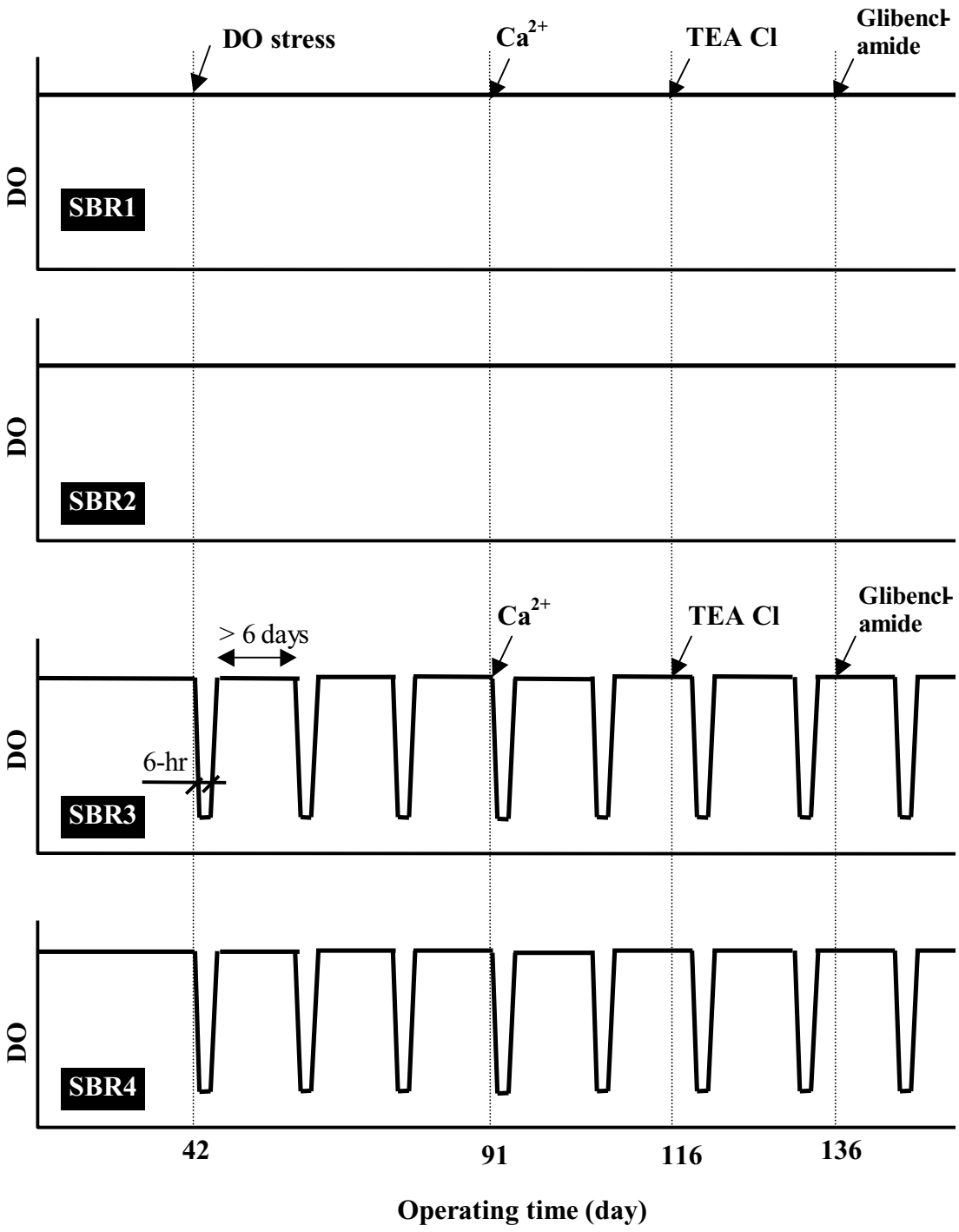


Figure 3.5 A schematic representation of the history of each SBR

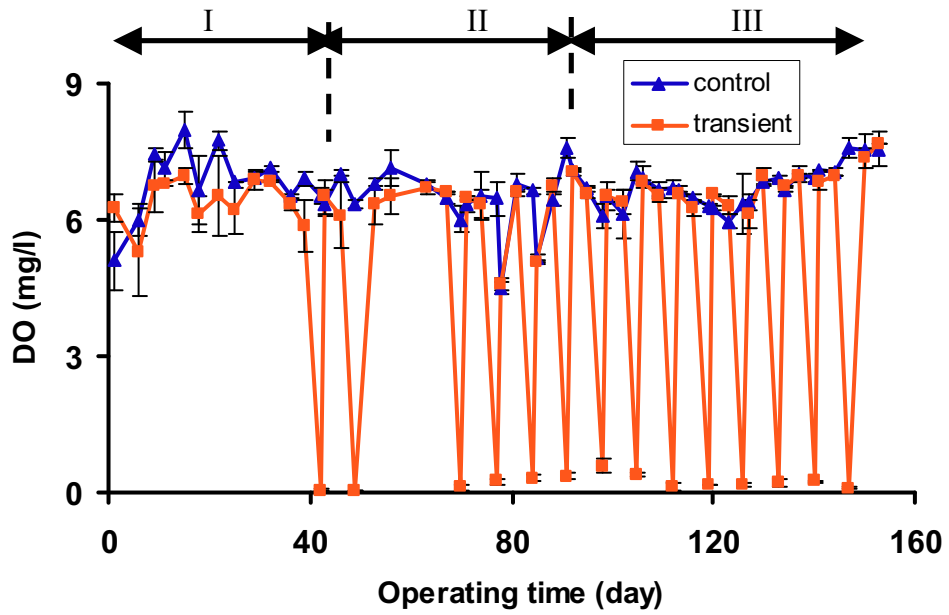


Figure 3.6 Dissolved oxygen (DO) in SBRs in the periods of acclimation (I), identifying deflocculation (II) and testing strategy (III). Control curve was the average DO values of SBR1 and SBR2 whereas transient curve was the average DO values of SBR3 and SBR4. Each data point represents the mean value with +/- one standard deviation.

Re-distribution of activated sludge samples from each SBR was performed before moving into next experimental period. Mixed liquor of activated sludge from each reactor was mixed and re-distributed at the end of acclimation period (period I) before moving into the stage of identifying deflocculation (period II). This was done to eliminate any difference produced over the acclimation period among reactor samples. After the pump accident, the mixed liquor of activated sludge from 4 reactors were re-mixed and re-distributed before the resumption of the DO transients. Similarly, mixed liquor samples from the control reactors SBR1 and SBR2 were re-mixed and re-distributed at the end of period II before moving into period III, so were the mixed liquor samples from the transient reactors SBR3 and SBR4. However, in the period III, prior to introducing a new chemical into the system (i.e., before adding TEA chloride and glibenclamide), no re-mixing/re-distribution of mixed liquor samples was performed. This was

to keep SBR2 and SBR4 as a pure reference to SBR1 and SBR3, respectively, throughout period III.

3.3.2 Sample Analysis

A summary of sample collection during one operating cycle is presented in Figure 3.7. All the parameters measured in batch experiments (Section 3.2.3) were analyzed in the continuous tests. The description of additional parameters examined at this stage is as follows:

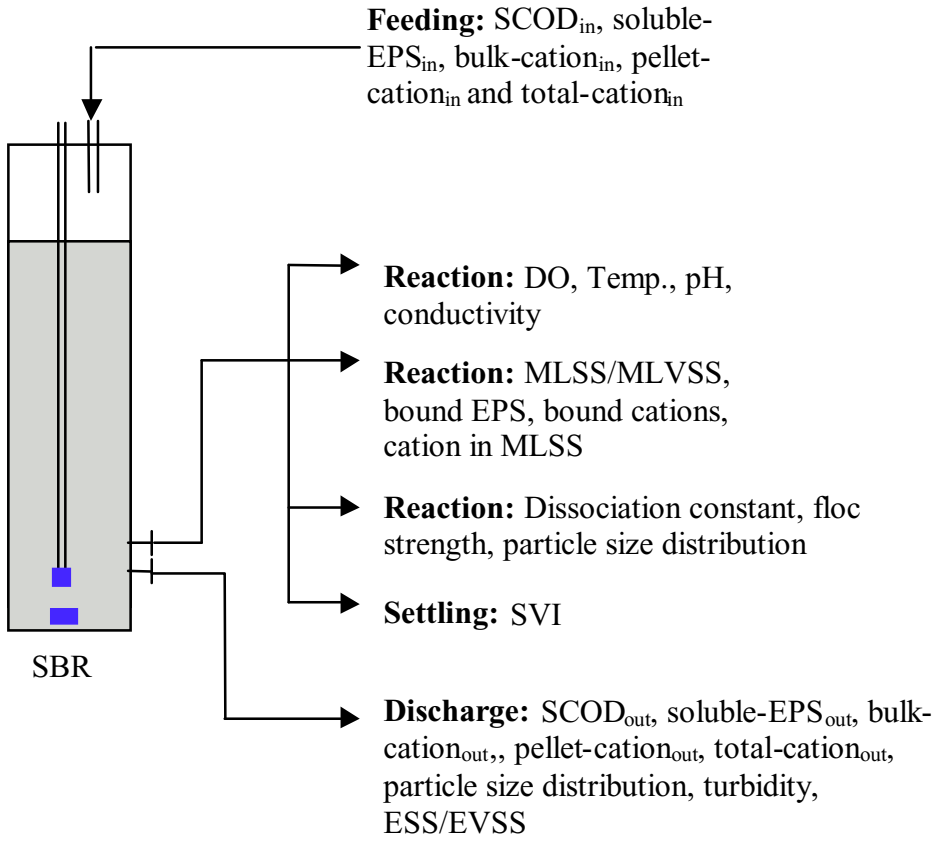


Figure 3.7 Data collection within an operating cycle from a SBR

Soluble COD: 30 mL of influent or effluent samples were filtered by 1.5 μm filter paper and the filtrate was collected for COD measurement, which was referred as soluble COD. COD measurement in the filtrates was conducted according to the Standard Methods (APHA, 1995, section 5220D): 2.5 mL of the filtered sample was added with 1.5 mL of digestion solution ($K_2Cr_2O_7$ + H_2SO_4 + $HgSO_4$) and 3.5 mL of sulphuric acid reagent (Ag_2SO_4 +H_2SO_4) in a HACH tube. The whole sample was digested at 150°C for 2 hours in a heat blocker (Model 45600-00, HACH Company, Loveland, Colorado). Absorbance reading was taken at 600 nm after the sample cooled down to room temperature. A calibration curve was made using potassium hydrogen phthalate (KHP) as a COD standard at different concentrations (212.5 mg dried KHP in 500 mL distilled water = 500 mg/L COD).

Sludge volume index (SVI): To identify DO effect on sludge compressibility, SVI (mL/g MLSS) was regularly monitored in each SBR during the settling phase of an operating cycle, as shown by the volume of activated sludge after 30 min of settling in proportion to the volume of sludge at the onset of settling cycle.

Cations analysis:

- Bulk cations in feeds (bulk-cation$_{in}$) and treated effluents (bulk-cation$_{out}$): 30 mL of feeds and treated effluents were collected during feeding and discharge cycles, respectively. After a high-speed centrifugation (10,000 g, 4°C and 15 min), the supernatants were taken for cation analysis, as described in Section 3.2.3.

- Cations in pellet (pellet-cation$_{in}$ and pellet-cation$_{out}$): The pellets from the above centrifugation were added with 1 mL of 10% HNO_3. Then, the solution was heated

at 100°C for 30 min before being diluted by 10 mL of 2% HNO_3. The samples were filtered and analyzed by ICP-AES.

- Cations in total (total-$cation_{in}$ and total-$cation_{out}$): 1 mL of feeds or treated effluents was added with 1 mL of 10% HNO_3, heated at 100°C for 30min., followed by a dilution with 10 mL of 2% HNO_3. The samples were filtered and analyzed by ICP-AES.

- Cations in MLSS: 1 mL of MLSS was added with 1 mL of 10% HNO_3, heated at 100°C for 30 min, followed by a dilution using 10 mL of 2% HNO_3. The samples were filtered and analyzed by ICP-AES.

- Cations in bound EPS: Same procedures as described in Section 3.2.3.

Particle size distribution and floc shear strength: From days 91 to 156, samples of treated effluents and mixed liquor were taken regularly for measuring particle size distribution and floc shear strength. The measurements were carried out by a Ph.D. student, Yuan Yuan. Detailed description of the methodology and procedures is presented by Yuan (2007). The particle size distribution was analyzed using a coulter particle size analyzer (Multisizer 3™, Beckman Coulter, Miami). The experiments on the shear strength of bioflocs were performed using a custom-made turbulent couette flow cell. Mixed liquor samples sizing from 45-63 μm and 75-90 μm were collected for the shear strength measurement. Since the control reactor SBR1 did not provide a sufficient amount of mixed liquor samples over the experimental time, the other three SBRs (SBR2 to SBR4) were used for monitoring the changes in shear strength. The effect of DO variations on floc shear strength was shown by the difference of average shear strength

between SBR2 and SBR4. The effect of extra Ca^{2+} on shear strength was demonstrated by comparing the average shear strength between SBR3 to SBR4. The average shear strength refers to the shear stress at which 50% of the tested bioflocs are broken up.

3.4 Batch Tests on *E. coli* Suspensions

The complexity of bioflocs in activated sludge presents challenges for further examining the proposed mechanism for sludge deflocculation. Activated sludge is mixed consortia of various microbial species dominated by prokaryotes. Compared to eukaryotic species, ion transport systems in prokaryotes are poorly studied. Types of membrane-bound ion transport systems present in activated sludge are unknown. Thus, it is difficult to identify which ion transport system(s) in activated sludge are involved in stress responses to O_2. To overcome this challenge, using a pure culture with identified membrane-bound ion transport systems to study responses to O_2 stress was carried out at this stage.

Despite being a facultative anaerobe, *E. coli* was chosen for the investigation of cation changes under O_2 stress. *E. coli* is a typical gram-negative prokaryote present in municipal activated sludge. Compared to other prokaryotic species, *E. coli* often serves as a model for studying membrane-bound ion transport systems in prokaryotes, for the ion transport systems in *E. coli* have been identified, including K^+ influx and efflux systems (Section 2.7.5.2). In this study, it was assumed that the presence of aggregates in *E. coli* suspensions was minimal. Thus, any changes in extracellular cations under the stress will be associated with cellular cation changes.

3.4.1 Culturing Conditions

E. coli K12 ER2925 was obtained from the New England Biolabs. Duplicate stabs of culture were made first as one stock culture (kept in dark at room temperature) and one subculture (i.e., working culture). A master agar plate was made by reviving a stored strain from the subculture stab and was kept at 4°C for a month. Luria-Bertani broth (Sigma-Aldrich Com., L2542) was used as a growth medium. A defined minimal medium was used to maintain cell cultures during the experiments, containing 25 mM glucose (Sigma-Aldrich Com., 99.5%), 6.6 mM Na_2HPO_4 (VWR Com., 99%), 0.3 mM KH_2PO_4 (EM Science, ≥99%), 3.6 mM NH_4Cl (Sigma-Aldrich Com., ≥99.5%), 0.4 mM $MgSO_4.7H_2O$ (Fisher Scientific, >99%) and 1.4 mM $CaCl_2.2H_2O$. All the media were sterilized by autoclave and were kept at 4°C for no more than a week.

An overnight culture: 10 mL of LB medium was first transferred into a sterile culture tube using a sterile pipettor, and was inoculated by a single bacterial colony from the master agar plate. The sample was cultured at 37°C, 60 rpm for an overnight (Ausubel, 1987).

Large volume of cultures: Twelve 250-mL sterile Erlenmeyer flasks, each containing 50 mL of LB medium, were used for growing large volume of cultures. Each flask was inoculated with 0.5 mL of overnight culture and was incubated at 37ºC and 300 rpm for 8 hours. The *E. coli* cultures were then centrifuged at 10,000 g for 10 min. The cell pellets were re-suspended into four 500-mL sterile Erlenmeyer flasks each containing 100 mL of fresh LB medium, to concentrate *E. coli* cells by 1.5-fold. The concentrated cultures were then incubated overnight at 37ºC and 60 rpm. The cell density reached approximately 5-9 x 10^9 CFU/mL. Viable cell

counts were carried out by serial dilutions (dilution factors: x 10^{-3}, x 10^{-3}, x 10^{-2}, and x 10^{-1}) and spreading on LB plates (Ausubel, 1987).

3.4.2 Experimental Approach

One hour before each experiment, *E. coli* cells were harvested by centrifugation at 10,000 g for 10 min, and were transferred into another four 500-mL sterile Erlenmeyer flasks containing 100 mL of defined minimal media described above. To allow the cells adapt to the new media and to create a well mixed homogenous suspension, the flasks were put into an incubator at 37ºC and 60 rpm for an hour.

Four to five 500ml Erlenmeyer flasks each containing 100 mL of *E. coli* cultures in defined minimal media were used for DO tests over 1.5-2 hours. The experiments were conducted in a laminar flow fume hood, to eliminate any airborne contamination during the experiments. Control samples (in duplicate flasks) were under sufficient aeration from a compressed air cylinder. Stressed samples (in duplicate or triplicate flasks) were purged by N_2 from a compressed N_2 cylinder. Time 0 was set as the onset of N_2 purging. DO concentrations in the stressed samples were maintained below 0.5 mg/L over the experiments. Samples were regularly taken for ion analysis (K^+, Na^+, Ca^{2+} and Mg^{2+}).

The effect of valinomycin on mediating K^+ efflux under O_2 stress was studied. As described previously, valinomycin is an antibiotic ionophore to actively transport K^+ across the cell membrane based on the electrochemical gradient of K^+. Separate tests on adding valinomycin (Sigma-Aldrich Com., 1 mg/mL solution in DMSO) were performed. At time 0 or after 30 min. of N_2 purging, valinomycin was introduced into one control and one or two stressed flasks, respectively, at a final concentration range of 4.5-9 μM.

3.4.3 Cation Analysis

Cations in bulk, in cell pellets and in total were analyzed. Sample treatments at this stage were modified from the ones described in Section 3.2.3: 1.3 mL of sample was taken from each flask every 30 min. To avoid the possible release of cellular K^+ into bulk solution during centrifugation, 0.2 mL of silicone oil AR 200 (Sigma-Aldrich Com.) was added into the sample before the centrifugation (Bakker, 1993). The total 1.5 mL of sample was then centrifuged at 13,000 g and 4ºC for 5 min. Cation concentrations (Na^+, K^+, Ca^{2+} and Mg^{2+}) in both supernatant and pellets were analyzed by ICP-AES.

3.5 Statistical Analysis

To get conclusive results, data from control and transient reactors at each time point were replicated. In the batch experiments on activated sludge, transient reactors were in triplicates, i.e., three independent reactors were operating under identical conditions. Thus, at each time point (except t=0), independent triplicate samples were taken from the triplicate reactors. In comparison, limited by the number of reactors and operating complexity, control samples were measured in duplicate (except Section. 4.1.2) taken from one control reactor. In this regard, the control duplicates were not independent. However, repeated runs were carried out on different days. This confirmed that experimental findings were of significance and reproducibility. In the continuous experiments, from days 42 to 90, independent duplicate reactors were operated under control conditions. The same were for the other two reactors as transient duplicates. In all the experiments performed in this study, sample measurements and preparations were in a randomized order to avoid any carry-over effects.

The statistical significance of the differences between control and transient samples was assessed by a paired two-sample t-test. The paired comparison is to group data in pairs with each comprising one measurement from one group. In this study, the pairing was based on experimental time. Thus, any time-related variance was excluded, including hour-to-hour variances in the batch experiments and day-to-day variances in the continuous experiments. In the batch experiments, during the DO limitation period (i.e., t = 2, 4 and 6), the control and transient samples were paired. The order of analyzing the control and transient samples within each pair was randomized. The difference between the control and transient data from each pair was calculated and was used for a two-sided t-test of significance. A cutoff p value for statistical significance was chosen as 0.05, i.e., any value of larger than 0.05 represents insignificant difference between the compared groups. Similarly, in the continuous experiments, control and transient samples were paired on transient days, and daily difference between the two groups was used for a significance test.

CHAPTER 4 EXPERIMENTAL RESULTS

The goals of the study were to identify the impacts of short-term low DO on treatment performance and properties of activated sludge, and to understand deflocculation of sludge under the DO transients. To achieve the goals, batch experiments and continuous experiments on activated sludge were carried out. Effects of the DO transients were demonstrated in terms of changes in effluent quality and the properties of bioflocs. Furthermore, batch tests on *E. coli* suspensions under the O_2 limit were performed, to examine the stress responses in a simple microbial system, and to support the mechanism understanding of deflocculation. Detailed results from each experimental stage are presented below.

4.1 Batch Experimental Results:

In a batch system, the effects of short-term low DO were studied with respect to the changes in turbidity, SS, EPS components and extracellular cations. Both soluble and bound EPS components were examined, including proteins, polysaccharides and humic substances. Concentrations of the EPS components and levels of bulk cations were measured in mg per litre of supernatant after centrifugation. A detailed description of sample collection and analysis is presented in Figure 3.4 and Sections 3.2.3.

4.1.1 Identifying the Effects of Short-term Low DO

4.1.1.1 Deflocculation of Activated Sludge

A low DO concentration (< 0.5 mg/L) caused a significant increase in supernatant turbidity (Figure 4.1). After 2-hour of N_2 purging, turbidity in the transient samples was approximately ten times higher than that in the control. The turbidity continuously increased under the oxygen

limitation. To explore the causes for increasing turbidity, the control and transient supernatants were observed under a microscope. It was found that the transient supernatant had much more suspended materials than the control, indicating that more cells or small bioflocs became suspended into the supernatant (Figure 4.2). As a consequence of the deflocculation, supernatant turbidity increased.

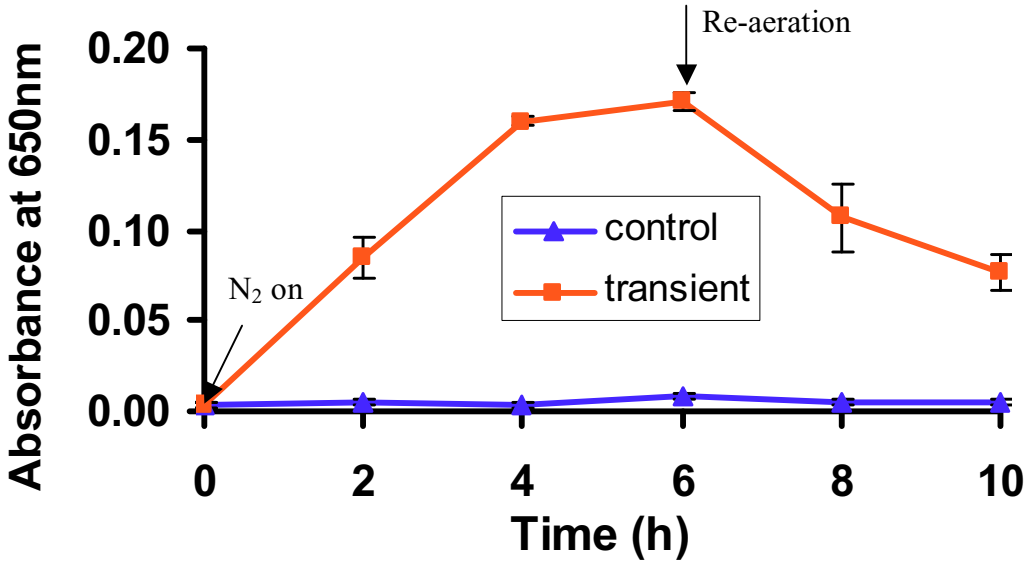
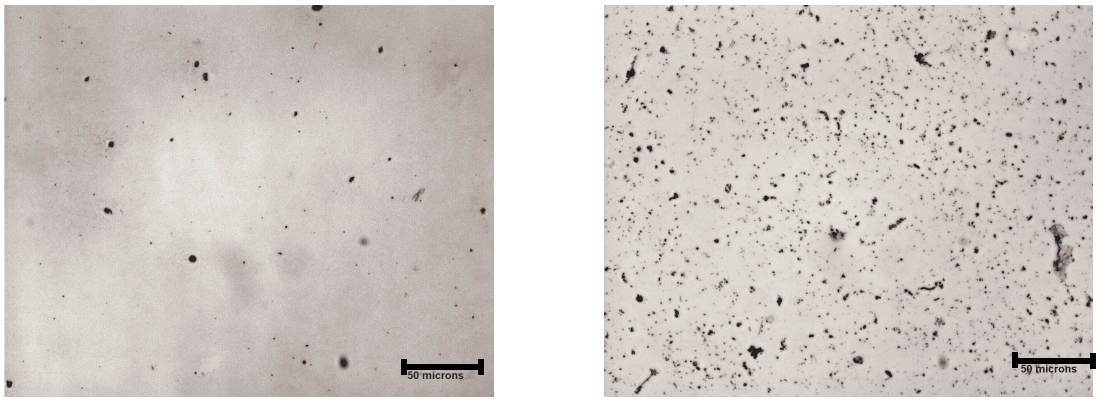


Figure 4.1 Typical turbidity changes under the DO transients: From t = 2 to 6 hours of low DO, turbidity in the transient supernatants was significantly higher than that in the control ones ($p = 2.9 \times 10^{-4}$). The error bars are +/- one standard deviation of turbidity measurements.



(a) (b)

Figure 4.2 Microscopic observation of supernatant (stage I): (a) control; (b) transient. The images were taken by Axiovert 200, inverted microscope. The bar represents 50 µm.

When aeration was restored in the transient reactors, a decrease in turbidity was observed (Figure 4.1). At t = 6 hours, aeration was re-supplied to the transient reactors and the DO concentrations increased up to 5.0 mg/L. The supernatant turbidity declined, as shown by the decreasing absorbance. Less suspended particles were observed under a microscope. This indicated a possible reflocculation when the stress was removed.

After 4-hour of N_2 purging, a plateau occurred in the turbidity profile of the transient samples from t = 4 to 6 hours (Figure 4.1). This indicated that the process of deflocculation slowed down to some extent even though the DO level continuously remained below 0.5 mg/L. The occurrence of plateau at t = 4 to 6 hours was consistently observed in repeated tests conducted on different days.

The average SS concentration in the transient supernatants (approx. 160 mg/L) was more than double that in the control (approx. 65 mg/L) at the end of N_2 purging (t = 6 hours) (Figure 4.3). The high transient SS concentration was attributed to unsettled cells, as supported by an

example of cloudy transient supernatant (Figure 4.4). The SS concentration in the transient supernatant decreased after re-aeration. All of these suggested that deflocculation occurred under DO limit and reflocculation occurred when the DO stress was removed.

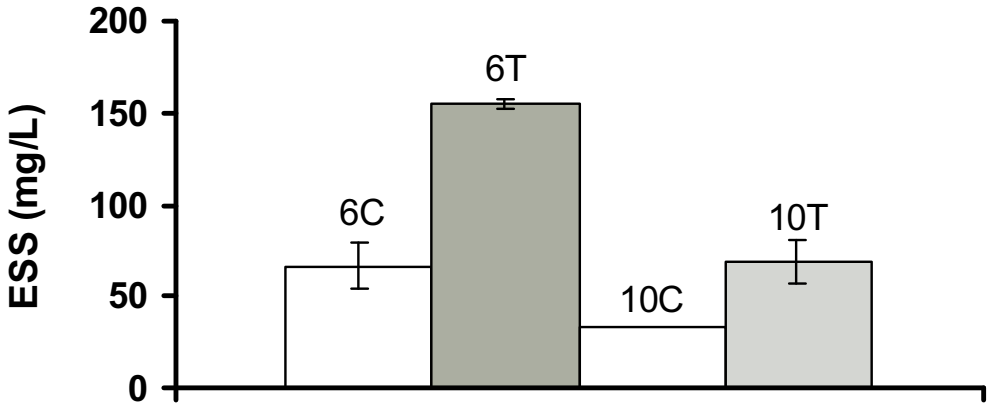


Figure 4.3 Typical changes in SS concentrations under DO transients: "6C" is the control sample at t = 6 hours (DO > 5 mg/L); "6T" is the transient sample at t = 6 hours (DO < 0.5 mg/L); "10C" is the control sample at t = 10 hours (DO > 5 mg/L); "10T" is the transient sample at t = 10 hours (DO > 5mg/L). The error bars represent +/- one standard deviation of SS measurements.

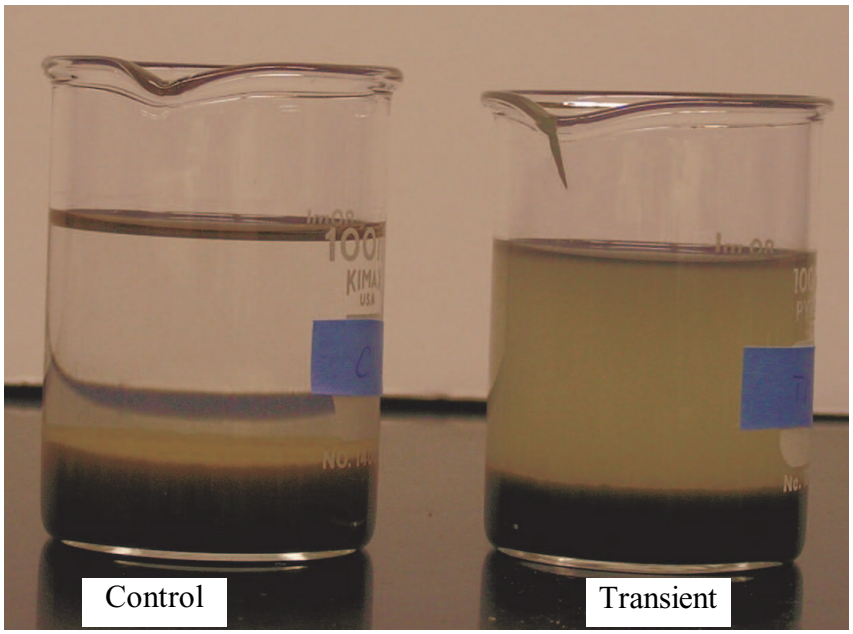


Figure 4.4 Image of supernatants at t = 6 hours: The transient sample had more cloudy supernatants than the control sample

4.1.1.2 DO Impacts on EPS Compounds

Significant increases in the levels of soluble proteins were observed under DO limitation. A typical experimental result is presented in Figure 4.5. Throughout the experiment, the concentrations of soluble proteins in the control samples remained low and stable (around zero). In contrast, soluble proteins in the DO-transient samples increased up to 30 mg/L from t = 2^{nd} to 6^{th} hour. In repeated experiments performed on different days, there was no "lag phase" in the profile of soluble proteins: the significant increases up to 10-20 mg/L of soluble proteins were observed within 2-hour of N_2 purging. In the last 4 hours (t = 6^{th} to 10^{th} hour), upon the reintroduction of oxygen, the level of soluble proteins dropped to a level close to that in the control samples.

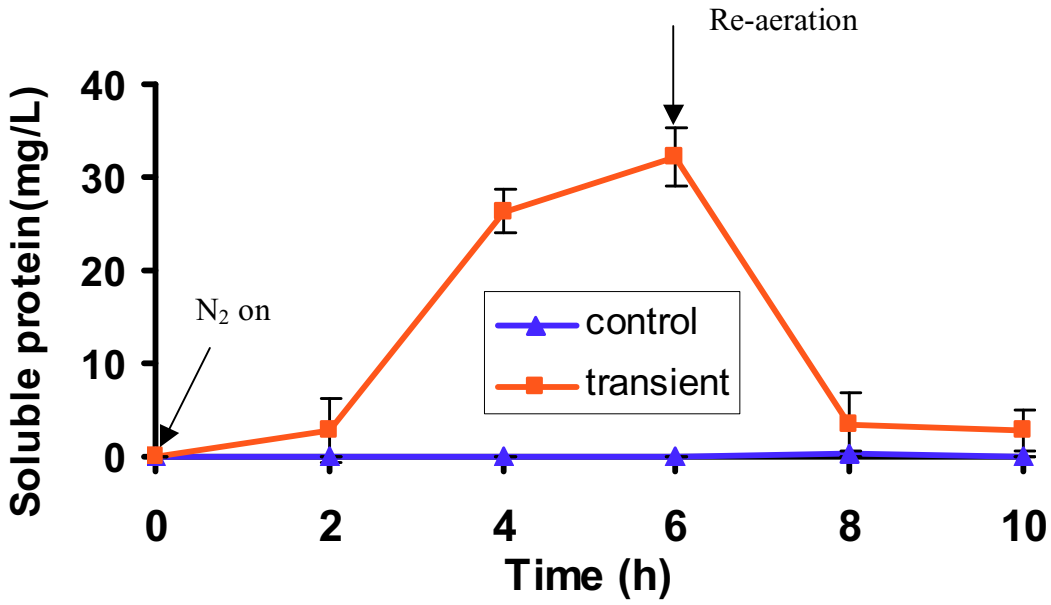


Figure 4.5 Typical changes in soluble proteins under DO transients: From t = 2 to 6 hours, soluble proteins in the transient samples were significantly higher than those in the control samples ($p = 2.3 \times 10^{-3}$). The error bars are +/- one standard deviation of bulk proteins.

From t = 2 to 6 hours, the concentrations of bound proteins in the transient samples (112 to 88 mg/L) were significantly lower than those in the control samples (143 to 107 mg/L), and both control and transient samples declined with time (Figure 4.6). The decreasing level of bound proteins under the DO disturbance suggests that bound proteins in EPS may be solubilised during the process.

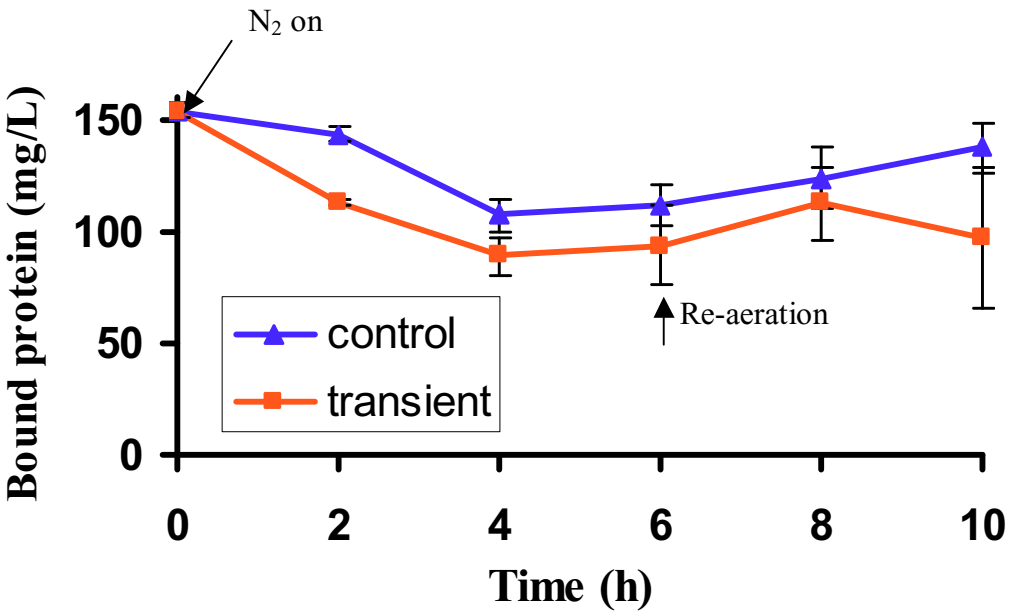


Figure 4.6 Typical changes in bound proteins under DO transients: From t = 2 to 6 hours, the transient samples had a lower concentration of bound proteins than the control samples (p = 0.035). The error bars are +/- one standard deviation of bound protein measurements.

Similar to the changes in soluble proteins, the release of humic substances into bulk phase was observed under DO limitation (Figure 4.7). At t = 6 hours, the average level of humic substances in the transient samples was approximately 4 times higher than that in the control sample. Reversible changes were observed once the DO levels were restored in the last 4 hours. Regarding the changes in the levels of humic substances in bound EPS, no significant difference was observed between the control and transient samples under DO limitation (Figure 4.8).

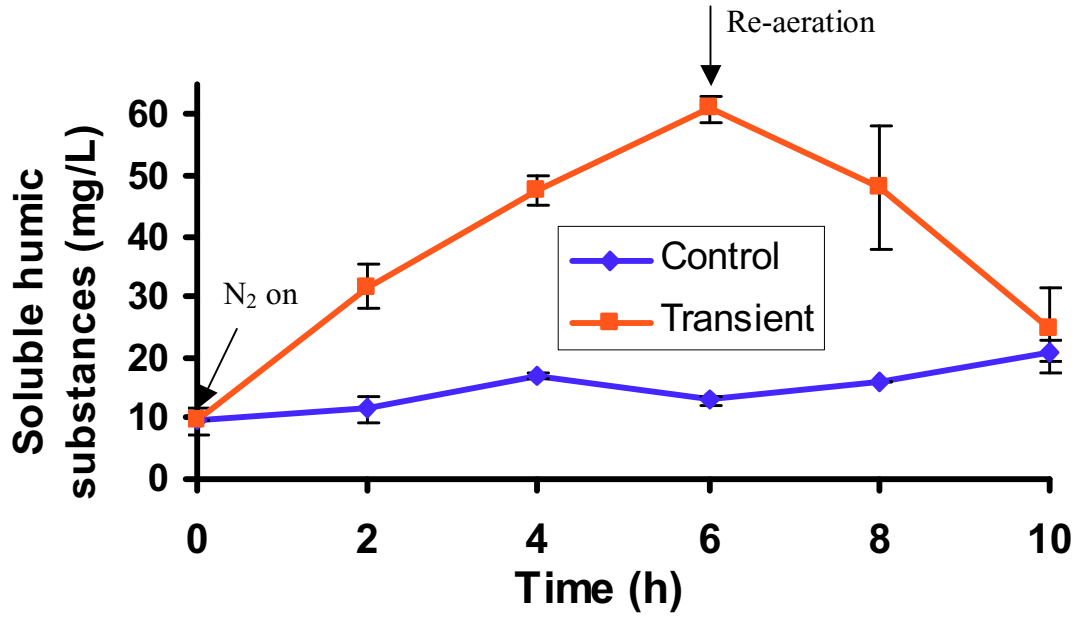


Figure 4.7 Typical changes in soluble humic substances under DO transients: From t = 2 to 6 hours, soluble humic substances in the transient samples were significantly higher than those in the control samples ($p = 6.3 \times 10^{-5}$). The error bars are +/- one standard deviation of bulk proteins.

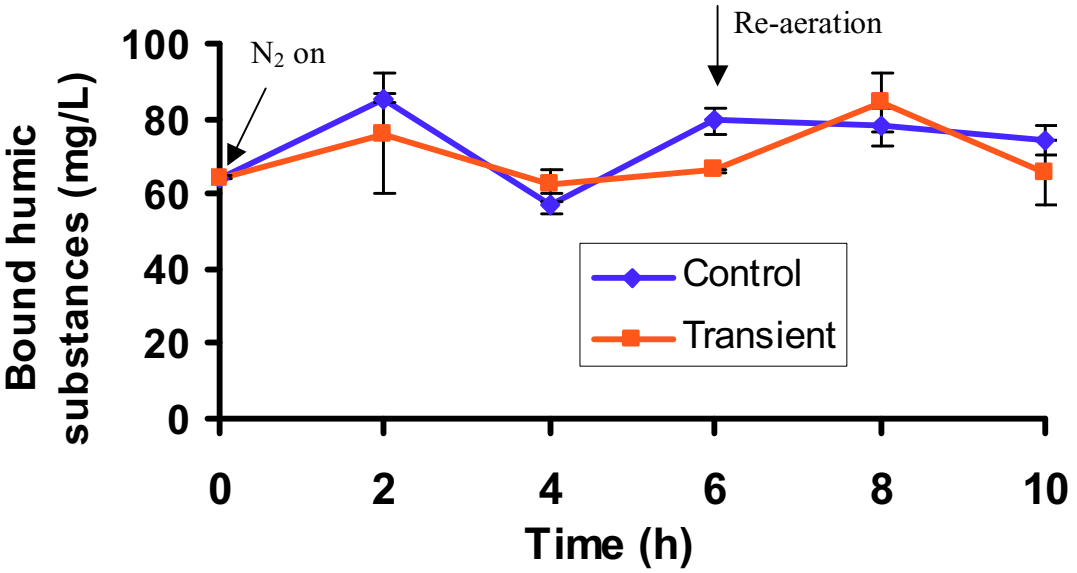


Figure 4.8 Typical changes in bound humic substances under DO transients: There were no significant differences between the control and transient samples from t = 2 to 6 hours (p = 0.18). The error bars are +/- one standard deviation of measurements.

In contrast to proteins and humic substances, soluble polysaccharides under DO disturbances were not significantly different from the control (Figure 4.9). In addition, the soluble polysaccharides increased with time even after re-aeration. This implied that the changes in soluble polysaccharides were not reversible upon the reintroduction of oxygen and were not related to the DO variations. Since no substrates/nutrients fed to the reactors throughout the experiments, the increasing levels of soluble polysaccharides were possibly related to endogenous metabolism of microorganisms. No significant changes in bound polysaccharides were observed between the control and transient samples throughout the experiments.

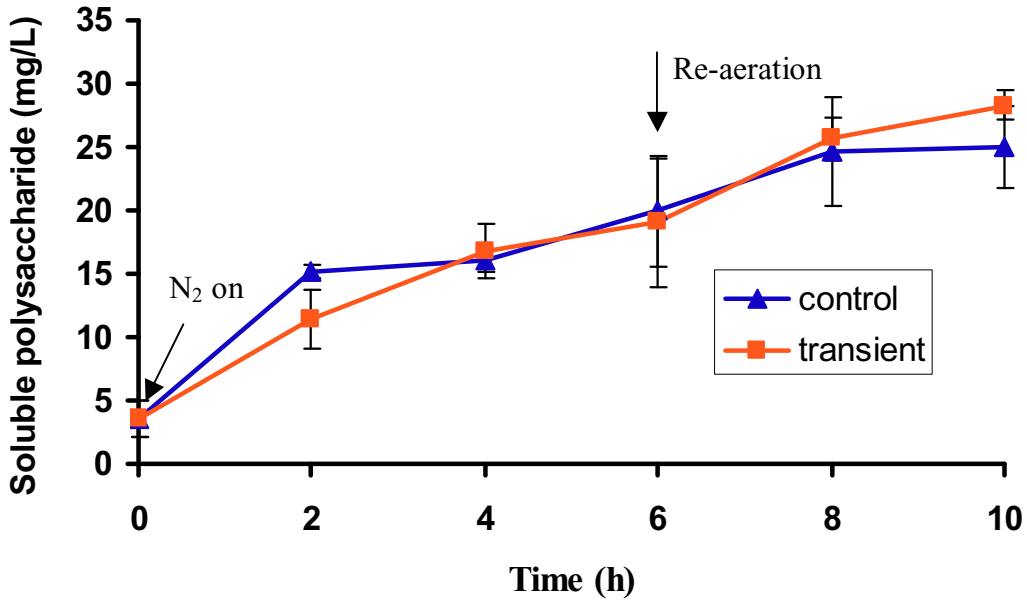


Figure 4.9 Changes in soluble polysaccharides under DO transients: There were no significant difference between the control and transient samples from t = 2 to 6 hours (p = 0.43). The error bars represent +/- one standard deviation of bulk polysaccharides.

4.1.1.3 DO Impacts on Extracellular Cations

Under DO limitation, bulk solution contained more K^+ (40% higher) and less Ca^{2+} (20-30% lower) (Figures 4.10 and 4.11). When the DO concentration was restored in the last 4 hours, the concentrations of bulk K^+ and Ca^{2+} returned closely to those in the control samples, suggesting reversible changes in the levels of K^+ and Ca^{2+} under the DO transient. In comparison, no significant changes in Na^+ and Mg^{2+} were observed under DO limitation (Appendix D).

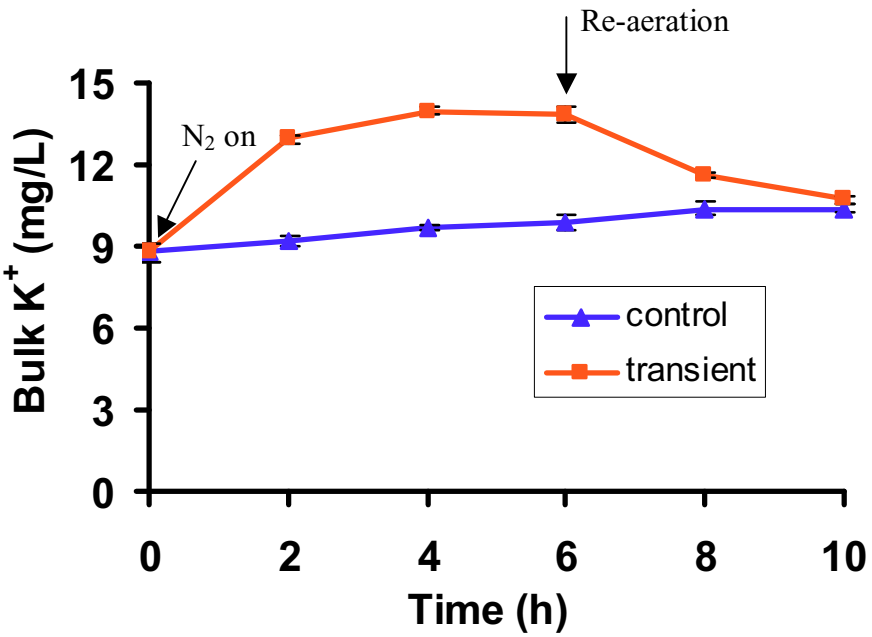


Figure 4.10 Typical changes in bulk K^+ under DO transients: From t = 2 to 6 hours, the transient samples had a higher concentration of bulk K^+ than the control samples ($p = 3.5 \times 10^{-7}$). The error bars are +/- one standard deviation of bulk [K^+] measurements.

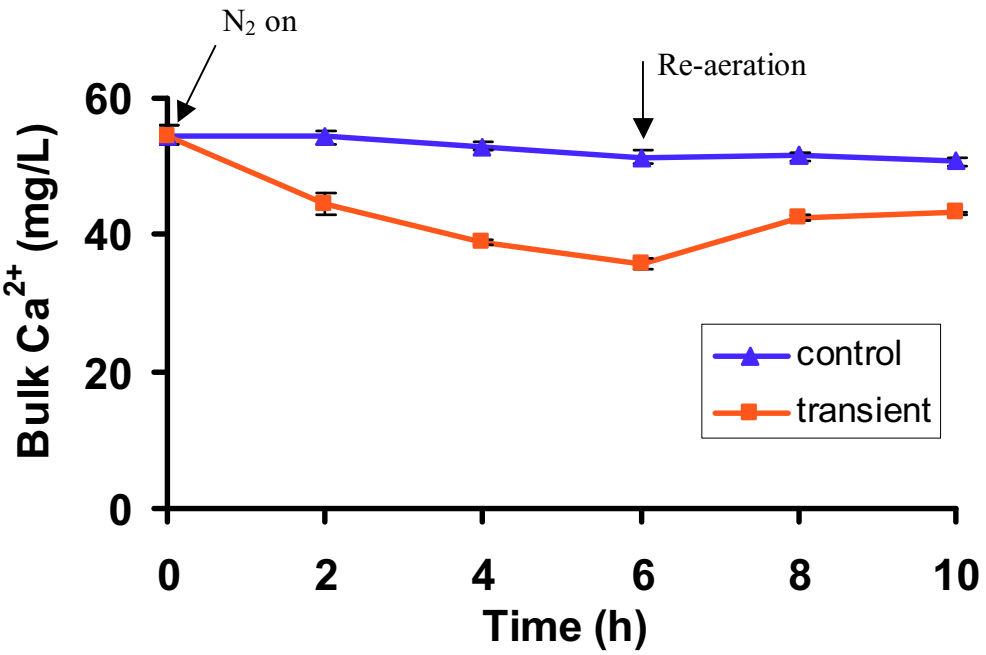


Figure 4.11 Changes of bulk Ca^{2+} under DO transients: From t = 2 to 6 hours, the transient samples had a lower concentration of bulk Ca^{2+} than the control samples ($p = 3.9 \times 10^{-5}$). The error bars are +/- one standard deviation of bulk [Ca^{2+}] measurements.

A negligible amount of extracellular bulk Ca^{2+} was re-associated with soluble EPS under DO limitation (Figure 4.12). Since the release of soluble EPS components (proteins and humic substances) were observed under DO limitation, it was necessary to clarify whether the reduction in bulk Ca^{2+} was associated with a possible re-binding between the released EPS components and bulk Ca^{2+}. Through acidification of soluble EPS, it was assumed that all the cations connected with EPS components were released. As shown in Figure 4.12, no significant changes in the Ca^{2+} level occurred before and after the acidification. Thus, the amount of Ca^{2+} re-combined with soluble EPS was negligible. In other words, the decreasing levels of bulk Ca^{2+} under the DO limitation was not attributed to the re-association with soluble EPS.

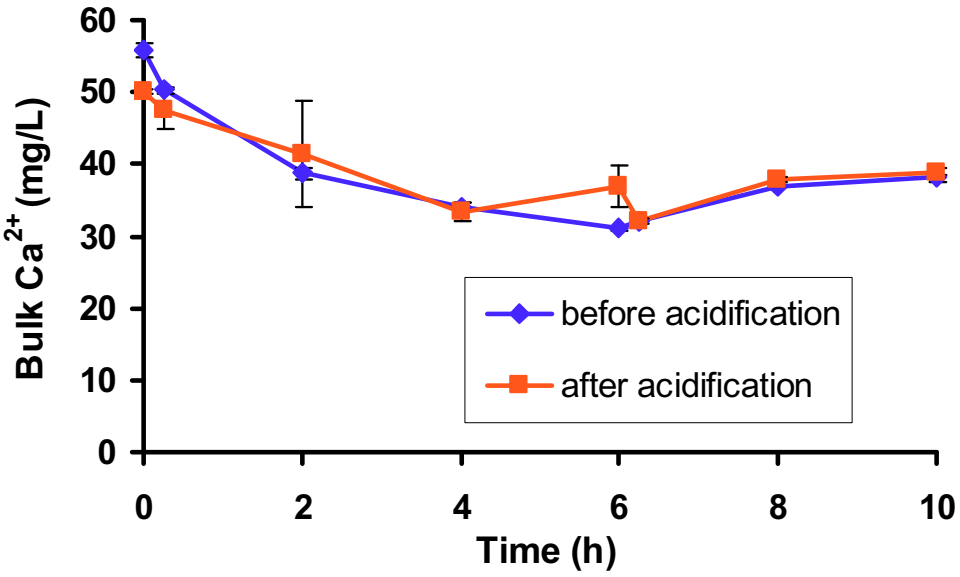


Figure 4.12 Changes of Ca^{2+} under acidification: There was no significant difference between the samples before and after acidification ($p = 0.59$). The error bars are +/- one standard deviation of [Ca^{2+}] measurements.

The cation levels in bound EPS were much lower than those in soluble EPS, and no conclusive results were obtained in comparing the concentrations of bound cations between the control and transient samples. Different from cations in bulk solution, ion concentrations in bound EPS (i.e., floc-associated cations) were much lower, even below the detection limit. Concentrations of Ca^{2+} and Mg^{2+} were below 0.8 mg/L (i.e., < 0.5 mg/g MLSS), and the level of K^{+} was below the detection limit of ICP-AES (< 2 mg/L). Na^{+} concentration ranges from 7-12 mg/L (i.e., 3 to 6 mg/g MLSS). Such a low amount of cations in the bound EPS suggests that most extracellular cations in mixed liquor could be extracted by a high-speed centrifugation (10,000 g). Thus, the cation levels in bulk solution became the focus in understanding and monitoring the sludge deflocculation under DO transients. On the other

hand, the low levels of bound cations may imply that EPS extraction efficiency by the CER method was low so that many floc-associated cations were unable to be removed.

Separate experiments on buffering pH showed that the DO limitation with pH maintained at 7.5 caused changes of the measurable parameters in similar magnitudes as those in the DO-stressed samples without pH buffering (Appendix E). In spite of an increment of pH under the DO limitation, increases in the turbidity, SS concentrations and the EPS components in bulk phase were mainly attributed to the DO transient rather than the pH increase. Thus, ignoring the effects of pH increment has little influence on the overall observations of DO impacts. In addition, extra Na^+ introduced into the system by adding the buffer solution may interfere with the observations on cation changes due to the DO limitation. Collectively, buffer solution was not further used in both batch and continuous experiments.

4.1.2 Preliminary Assessment of Mechanisms

4.1.2.1 External Addition of K^+ and Ca^{2+}

Extra K^+ and Ca^{2+} were added into the batch system, to test whether deflocculation could be triggered by adding K^+ in a comparable amount to the increase of bulk K^+ under the DO limit. As well, it was to test if the presence of extra Ca^{2+} could prevent the decline in bulk Ca^{2+} under DO stress and thereby preventing deflocculation.

4.1.2.1.1 Effects of Extra K^+

In this test, extra K^+ as KCl was added into one aerated reactor. Each dataset of control and KCl-added samples were measured in duplicates that were from the same reactor. Transient samples were in duplicates from two independent repeated reactors. Compared to the control

and transient reactors, results from the KCl-added reactor showed whether the extra addition of K^+ caused sludge deflocculation at a similar extent to the deflocculation under DO limit.

Regarding turbidity changes, external addition of K^+ did not lead to sludge deflocculation (Figure 4.13). Despite an extra amount of K^+ in the KCl-added reactor, deflocculation only occurred in the reactors exposed to low DO, shown by the turbidity being more than 10 times higher than in the other reactors.

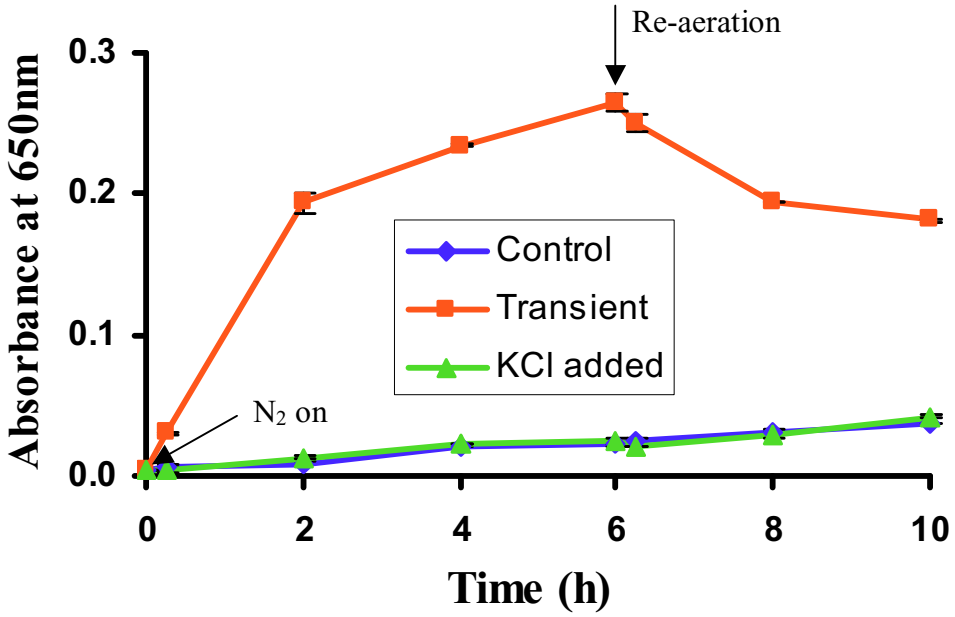


Figure 4.13 Turbidity changes under the addition of KCl: The samples exposed to a DO limitation had the highest turbidity. There was insignificant difference between control samples and KCl-added samples ($p = 0.18$). The error bars are +/- one standard deviation of turbidity measurements

External addition of KCl increased the amount of K^+ in the solution but had no impact on the level of Ca^{2+}. The amount of K^+ in soluble solution of the KCl-added reactor was 50% higher than that in the DO transient reactors (Figure 4.14). However, the level of Ca^{2+} in the

KCl-added reactor remained similar to that in the control air-aerated reactor (Figure 4.15). Thus, only a high level of bulk K^+ at current range did not cause deflocculation.

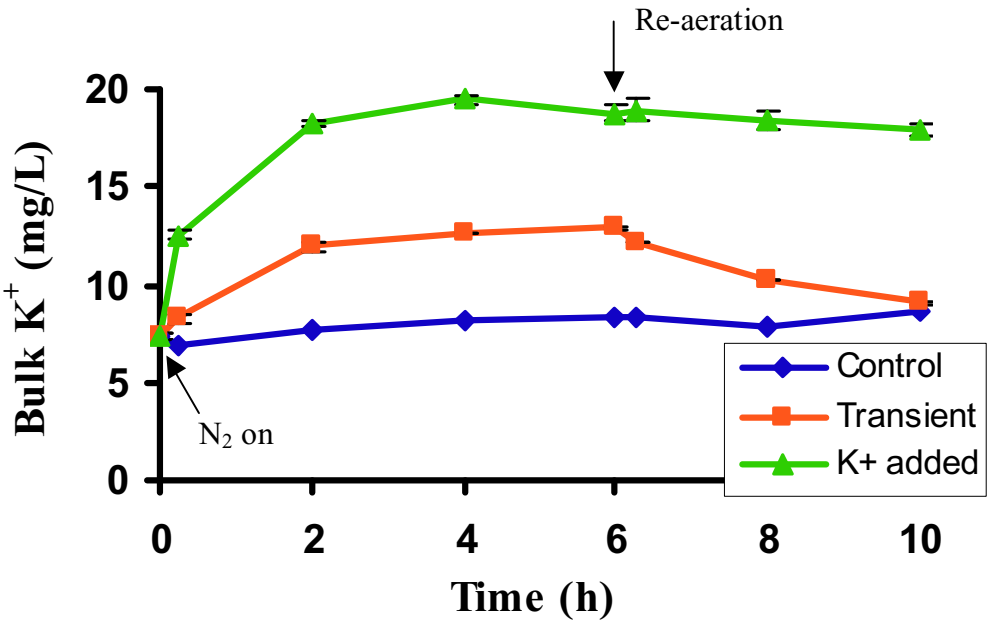


Figure 4.14 K^+ in the soluble solution under the addition of KCl: Addition of KCl caused the highest level of bulk K^+, higher than the K^+ increment due to DO limitation. The error bars are +/- one standard deviation of bulk [K^+] measurements.

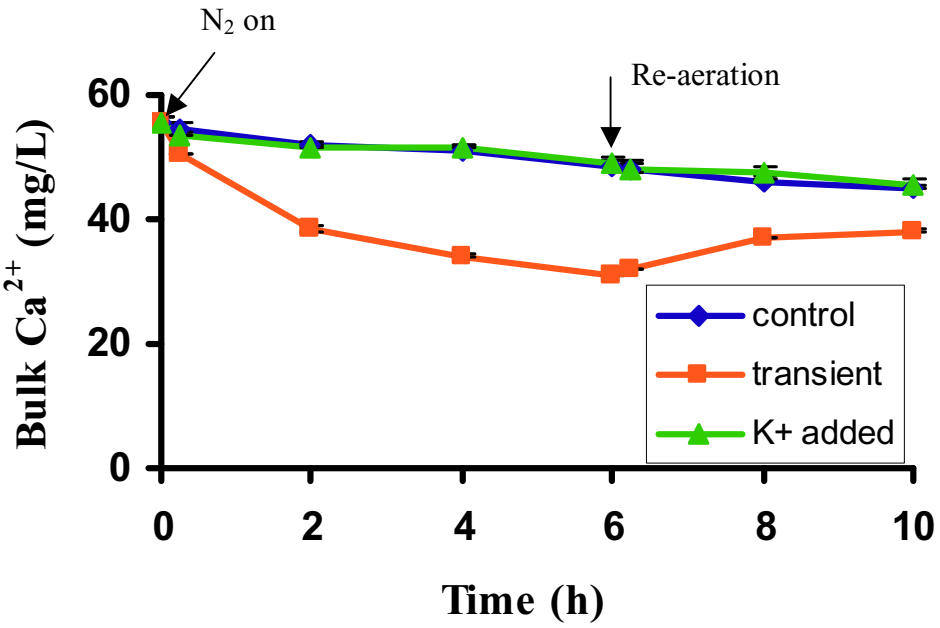


Figure 4.15 Ca^{2+} in the soluble solution under the addition of KCl: The samples exposed to DO limitation had the declining levels of bulk Ca^{2+}. There was insignificant difference between control samples and KCl-added samples ($p = 0.29$). The error bars are +/- one standard deviation of bulk [Ca^{2+}] measurements.

Changes in turbidity and bulk cations under DO limitation were observed within 15 min after the onset of N_2 purging. At t = 15 min, transient turbidity increased by 5 times, bulk K^+ in the transient solution increased by 12% and bulk Ca^{2+} in the transient solution declined by 9% (Figures 4.13 to 4.15). After 15 min of re-aeration in the transient reactors, transient turbidity declined by 6%, the level of K^+ decreased by 5% and the level of bulk Ca^{2+} increased by 3%. Changes in these parameters within a short time implied that cell responses to a short-term low DO could be a physiological response at the molecular level, rather than a shift in microbial population.

4.1.2.1.2 Effects of Extra Ca^{2+}

Extra Ca^{2+} as $CaCl_2.2H_2O$ was added into one aerated reactor and one transient reactor to test whether the addition of Ca^{2+} could prevent deflocculation under the short-term low DO. Limited by the number of reactors, each datapoint was measured in duplicates from the same reactor.

Turbidity results showed that deflocculation occurred in the N_2-purging reactors regardless of the addition of Ca^{2+} (Figure 4.16). Extra Ca^{2+} had no effect on the system, as shown by insignificant difference between the DO-transient reactors (labelled as "Transient" and "Transient_Ca2+").

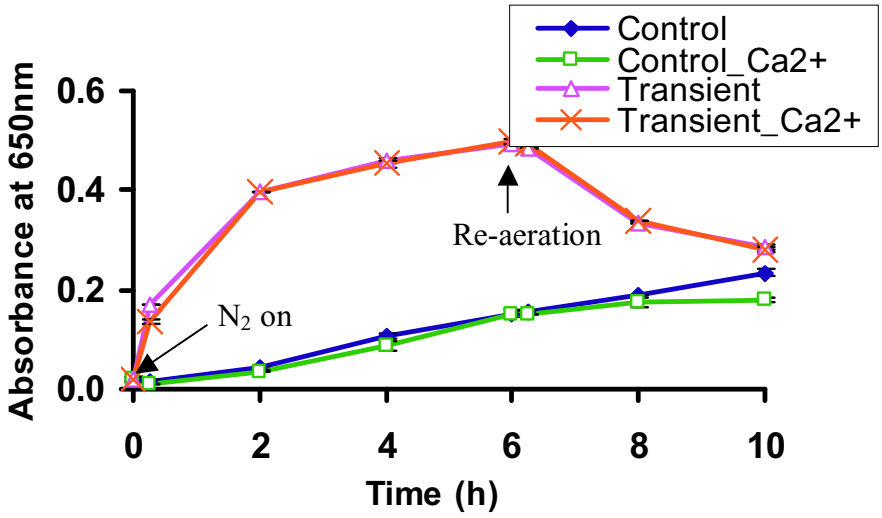


Figure 4.16 Changes of turbidity under the addition of $CaCl_2$: The samples exposed to DO limitation had the highest turbidity. Regardless of extra Ca^{2+}, there was no significant difference between turbidity in the transient reactors ($p = 0.36$). The error bars are +/- one standard deviation of turbidity measurements.

The levels of bulk K^+ continuously increased under DO limitation regardless of the presence of extra Ca^{2+} (Figure 4.17). Both transient reactors with and without extra Ca^{2+}

contained a higher amount of K^+ than the air-aerated reactors, and then returned to the control level after the restoration of oxygen.

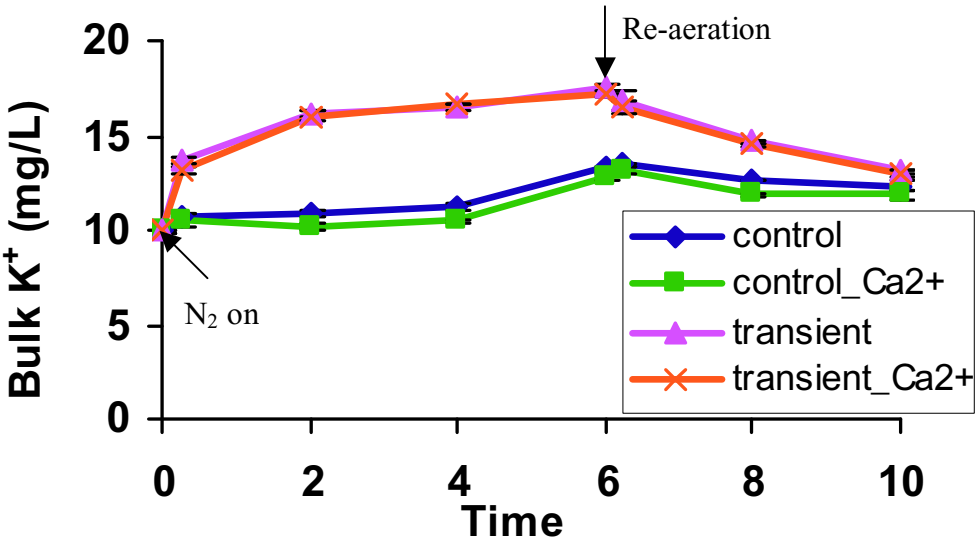


Figure 4.17 Changes of K^+ under the addition of $CaCl_2$: Regardless of extra Ca^{2+}, the samples from both transient reactors had a higher level of K^+ than those from control reactors under DO limitation. The error bars are +/- one standard deviation of bulk [K^+] measurements.

The levels of bulk Ca^{2+} in both transient reactors tended to decline under the DO limitation whereas the amount of Ca^{2+} in the air-aerated reactors remained relatively stable (Figure 4.18). Regardless of extra $CaCl_2$ introduced into one transient reactor (labelled as “transient_Ca2+”), the level of bulk Ca^{2+} kept on decreasing by 26% from t = 15 min. to 4 hours (denoted by "x"), implying a continuous loss of bulk Ca^{2+} under the DO stress. This suggested that one-time addition of Ca^{2+} was not sufficient to prevent deflocculation.

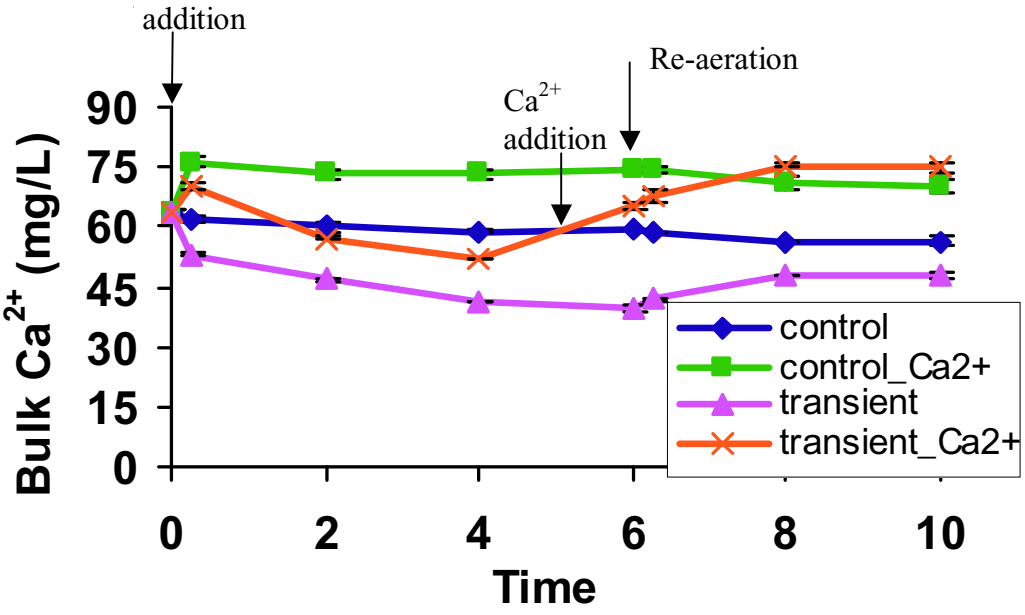


Figure 4.18 Changes of Ca^{2+} under the addition of $CaCl_2$: Extra Ca^{2+} was introduced at t = 0 and t=5 hours. In spite of adding Ca^{2+}, the samples from transient reactors had declining levels of bulk Ca^{2+} under DO limitation. The error bars are +/- one standard deviation of bulk [Ca^{2+}] measurements.

Similar to the aforementioned time scale, distinct changes in turbidity, levels of bulk K^+ and Ca^{2+} under the DO limitation were observed within 15 min of DO limitation, as shown by 7-fold increase in turbidity, 37% increase in bulk K^+ and 16% decrease in bulk Ca^{2+}. This again indicated a possible metabolic response to the short-term low DO.

4.1.2.2 Analysis of Time Constants

Preliminary analysis of the time scales (e.g., time constants) of the changes in bulk cations, turbidity and EPS compounds did not clarify the cause-and-effect relationship among these responses. It was assumed that a DO stress, as a pulse disturbance, was applied to a first-order system (Sample calculations are shown in Appendix F). For different experiments, the time constant of turbidity profile varied from 1.5-2.5 hours, while the time constant of soluble EPS

compounds (proteins and humic substances) were approximately 1.8-2.5 hours, and the time constant of bound EPS proteins was 1.5-2 hours. For K^+ and Ca^{2+} in bulk, the time constants were estimated as 1.5-1.8 hours and 1.8-2 hours, respectively. Collectively, there was no significant difference between the time constants of these responses. However, the magnitude of these time constants (i.e., hours) suggests that responses to a short-term low DO disturbance should not simply be a physical or chemical response, for the latter one usually occurs within a shorter time scale. Furthermore, significant changes in bulk K^+/Ca^{2+}, turbidity and soluble EPS compounds were observed after 15 min. of DO limitation. This suggests that probing the time scale of the DO responses within a shorter time span (e.g., min.) is valuable for a better understanding of deflocculation.

4.1.2.3 The Roles of Proteins and Polysaccharides in Preventing Sludge Deflocculation

Enzymatic tests were carried out to degrade EPS proteins and polysaccharides individually, and to assess their functional roles in sludge deflocculation. Turbidity and the concentrations of proteins and polysaccharides were measured in duplicate at each time point.

Turbidity in the trypsin-treated sample was 4 times higher than that in the control after 20 min, and was 6 times higher than that in the control after 2 hours (Figure 4.19). Microscopic observation showed the increasing number of suspended particles in trypsin-treated samples (Figure 4.20). Since trypsin degrades proteins, the substantial increase of turbidity indicated that proteins were a key factor in preventing sludge deflocculation. In addition, compared to the previous turbidity results from DO limitation (0-6 hours), the increasing turbidity caused by trypsin had a similar magnitude as that under DO limitation in the first 4 hours (Figure 4.19).

Thus, it was reasonable to speculate that deflocculation under the DO limitation might be related to the changes in EPS proteins.

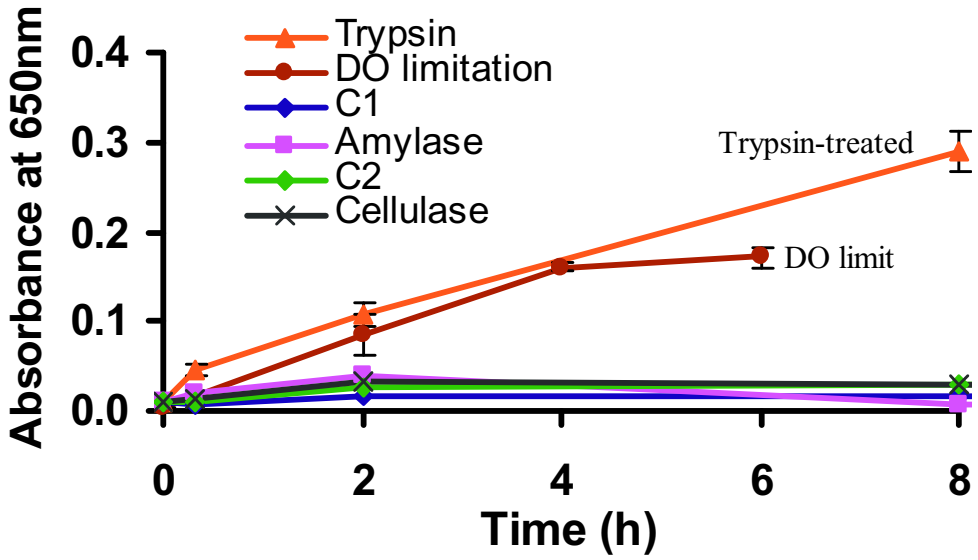
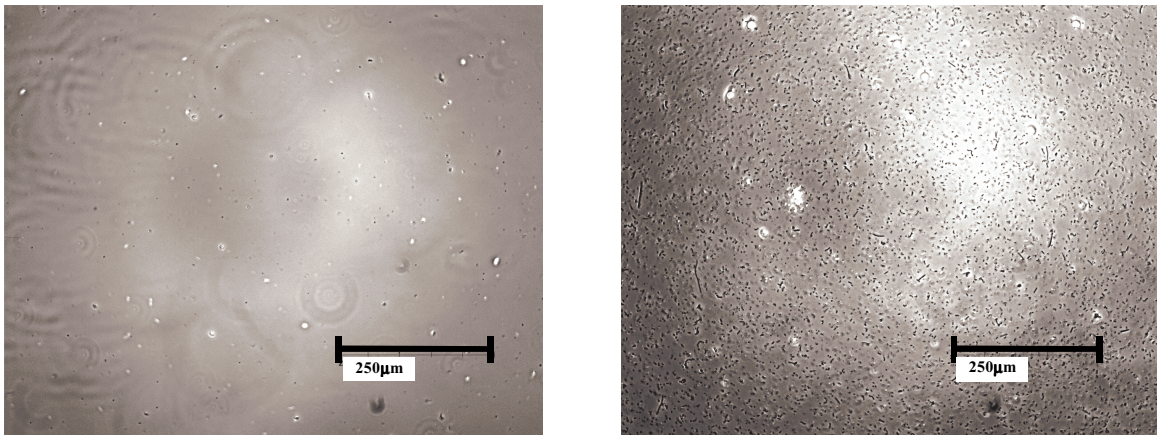


Figure 4.19 Turbidity changes in enzyme tests: Operating temperatures for trypsin and cellulase were 37ºC, for amylase was 25ºC. Two reference samples were prepared at 25ºC (C1) and 37ºC (C2), respectively. The trypsin-treated samples had the highest turbidity increment over the time. The error bars represent +/- one standard deviation of turbidity measurements.



(a) (b)

Figure 4.20 Microscopic observation of supernatant in trypsin test: (a) control; (b) transient. The image was taken 8 hours after the addition of trypsin by Axiovert 200, inverted microscope. The bar represents 250 µm

There were insignificant changes in turbidity when EPS polysaccharides were degraded by cellulose and amylase (Figure 4.19). After 20 min to 8 hours, the changes in turbidity of the sludge treated with cellulase or amylase were negligible. Thus, polysaccharides appeared to be less involved in preventing sludge deflocculation.

By analyzing the concentrations of proteins and polysaccharides in the enzyme-treated samples, it supported that proteins acted as a glue-like component to hold EPS matrix. In trypsin-treated samples, a high amount of protein (> 250 mg/L) was released after 2-hour digestion, accompanied by a high level of polysaccharides (> 80 mg/L). In comparison, in the sample treated with amylase and cellulose for 2 hours, only a trace of protein (< 6 mg/L) was present whereas the amounts of polysaccharides were above 500 mg/L and 100 mg/L, respectively. Accordingly, it was suggested that proteins were a key component to bridge

polysaccharides with other compounds or cells. The degradation of proteins would lead to a release of polysaccharides.

Following the enzymatic tests, experiments on identifying the profiles of EPS proteins (both bound and soluble proteins) under DO limitation were performed using SDS-PAGE. One hypothesis in this research is that deflocculation of activated sludge is the consequence of the degradation of EPS proteins by specific extracellular enzymes released or activated under DO stress. Thus, the SDS-PAGE tests were to examine the possible occurrence of specific extracellular enzymes. However, results show that the expression of EPS proteins on gels was not clear due to the low concentrations of proteins (< 100 mg/L). Usually, a concentration of 1 mg/mL is required for a clear gel expression. Therefore, effort has to be made on pre-treatment to increase the protein concentrations, and on optimization of the analytical techniques (e.g., buffer solution, staining reagents, sample loading volumes). Yang (2005) provided a detailed description of these tests.

4.1.3 Summary of Batch Experiments

Batch studies showed deflocculation of activated sludge under transients of short-term low DO, as characterized by significant increases in supernatant turbidity and SS levels, the release of soluble EPS proteins and humic substances. There were consistent increases in bulk K^+ and decreases in bulk Ca^{2+} under the DO limitation, but the relationship between the cation changes and deflocculation remains unclear.

The focus of fundamental understanding of deflocculation in the following chapters is on the examination of extracellular cations under the DO limitation. Though the importance of EPS proteins in holding bioflocs together was demonstrated in enzymatic tests, there was

incomplete success in exploring the protein profile using SDS-PAGE. On the other hand, there was a clear correlation between the deflocculation and changes in extracellular cations under DO transients. It is worth further exploring whether there is a causal relationship between the cation changes and deflocculation.

4.2 Continuous Experimental Results

The continuous experiments aimed to examine DO transients on treatment performance, to understand deflocculation and to explore the solutions for minimizing deflocculation. The impacts of short-term low DO were assessed by comparing the performance of duplicate control reactors with that of duplicate transient reactors in the first 90 days. From days 91 to 156, different strategies for deterring the deflocculation were tested, including separate addition of Ca^{2+}, TEA chloride and glibenclamide into the daily feeds of one control and one stressed reactors. Each chemical was tested for a period of 3 weeks. Experimental results are presented in detail below.

4.2.1 Identifying Deflocculation (Days 1-90)

4.2.1.1 Operating Performance under the DO Transients

From days 42 to 90, DO limitation induced a significant reduction in the removal of SCOD by more than 50% (Figure 4.21). Once the DO limitation was removed, average removal efficiency of SCOD in the transient reactors returned to the similar level as that in the control SBRs. This implies that the changes in SCOD removal are correlated with DO variations.

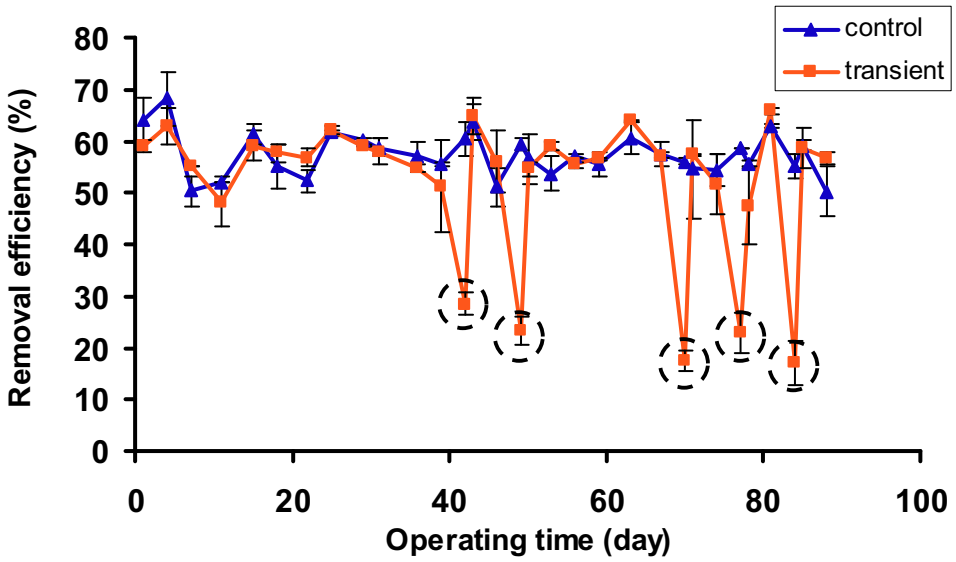


Figure 4.21 Removal of SCOD under DO transients: SCOD removal in the control reactors remains stable throughout the experiments. In the transient reactors, removal of SCOD significantly decreased from approximately 60% to less than 30% ($p = 6.2 \times 10^{-6}$). Transient days are days 42, 49, 70, 77 and 84, as marked by cycles. Each data point represents the mean value with +/- one standard deviation.

A further examination on the SCOD profile within an 8-hour operating cycle showed that the removal of SCOD mainly occurred in the first 120 min (Figure 4.22). After this, SCOD in the control reactors remained relatively stable, whereas SCOD in the transient reactors increased by more than 10% from time t = 160 min to 480 min. The increasing SCOD in the transient reactor suggested that some biopolymers from EPS were released under the DO disturbance, promoting the increases in SCOD of the treated effluents.

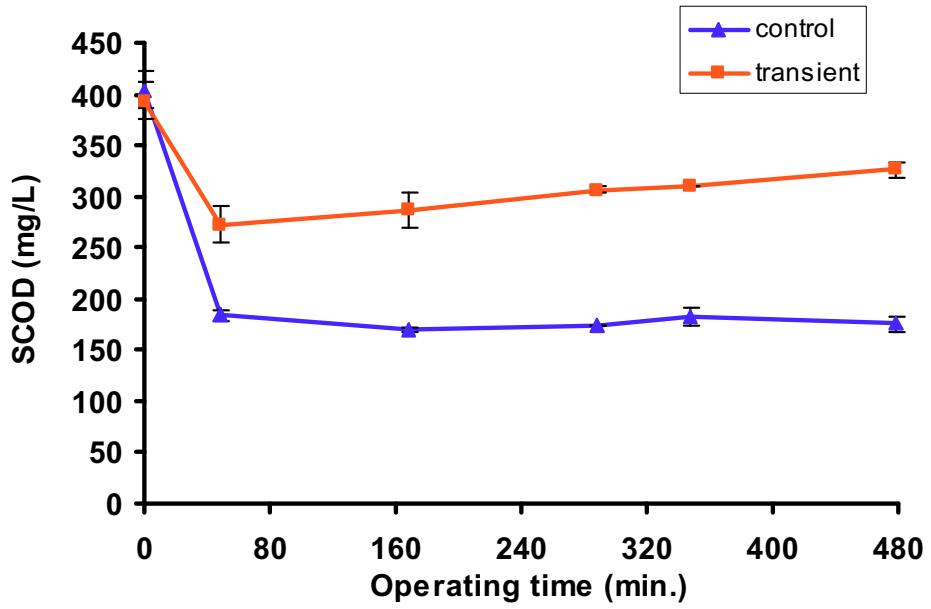


Figure 4.22 SCOD profile in an 8-hour operating cycle: transient reactors had a higher SCOD than control ones. Average transient SCOD increased by 13% from t = 160 to 480 min. Each data point represents the mean value with +/- one standard deviation.

Effluent turbidity increased by more than 100% under DO limitation, indicating the occurrence of sludge deflocculation (Figure 4.23). The supernatant for turbidity measurement was examined under a microscope after being gram-stained. More suspended particles (cells and small flocs) were observed from the transient supernatant, confirming that the increasing turbidity was caused by increasing suspended particles. After the restoration of oxygen concentration, effluent turbidity was able to return to the control levels within 24 hours.

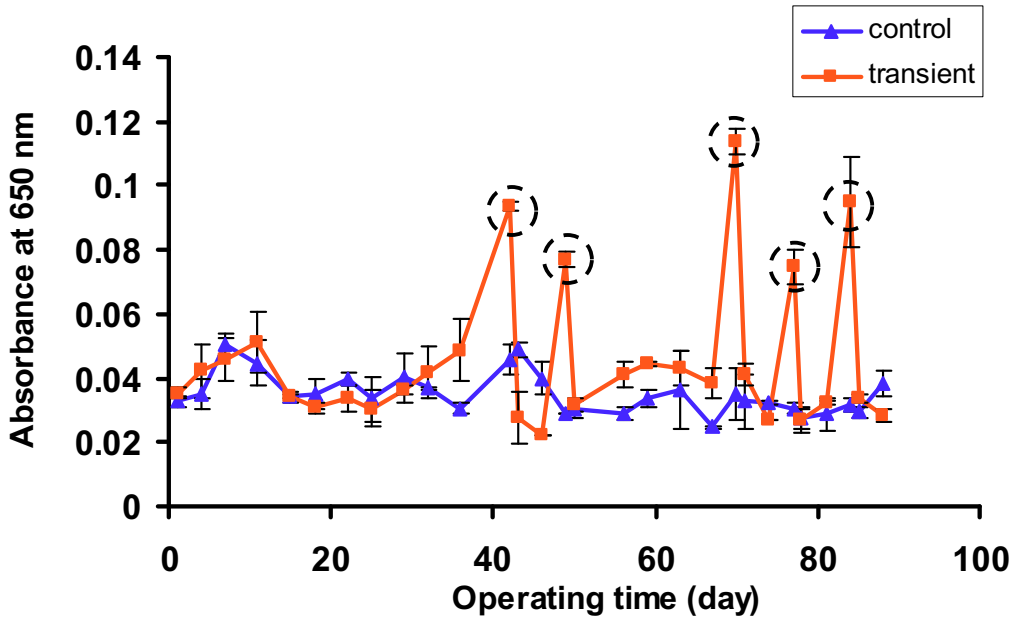


Figure 4.23 Changes in effluent turbidity under DO transients: transient turbidity (as marked by cycles) was significantly higher that in the control reactor on transient days 42, 49, 70, 77 and 84 ($p = 9.4 \times 10^{-4}$). Each data point represents the mean value with +/- one standard deviation.

Similarly, despite the relatively large variations, a substantial increase of ESS by 100-200% was observed under DO limitation, followed by a reversible change upon the restoration of DO (Figure 4.24).

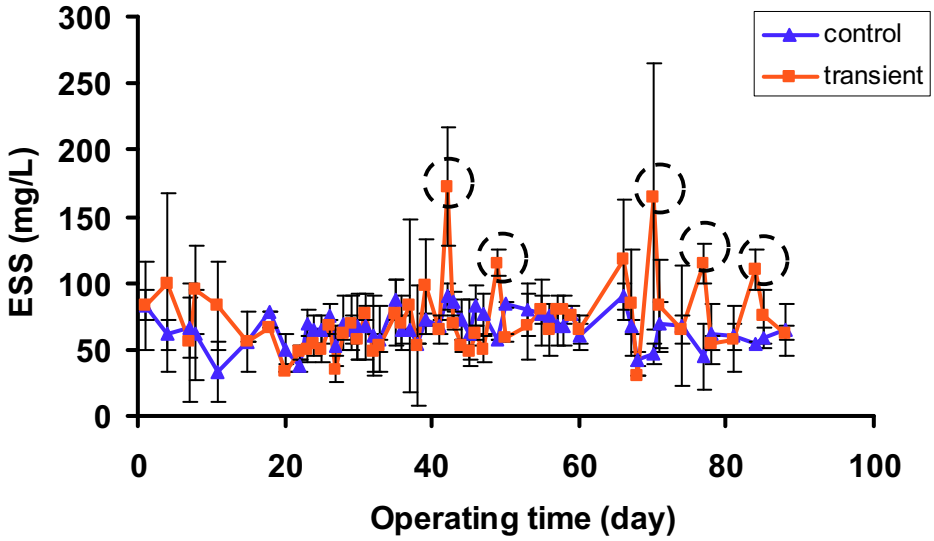


Figure 4.24 Effect of DO transients on ESS: Despite relatively high variations in the measurements, ESS concentrations in the transient reactors (as marked by cycles) were significantly higher than those in the control reactors on transient days 42, 49, 70, 77 and 84 ($p = 2.6 \times 10^{-3}$). Each data point represents the mean value with +/- one standard deviation.

The negative impacts of DO limitation were also demonstrated by a reduction of removal of humic substances from 40% to below 20% (Figure 4.25). Daily feeds to the SBRs contained approximately 200-300 mg/L humic substances, 20-30 mg/L polysaccharides and undetectable amount of proteins. The high amount of humic substances was attributed to the origin of wastewater, i.e., from a pulp and paper mill. Under DO limitation, more humic substances were left in the treated effluents.

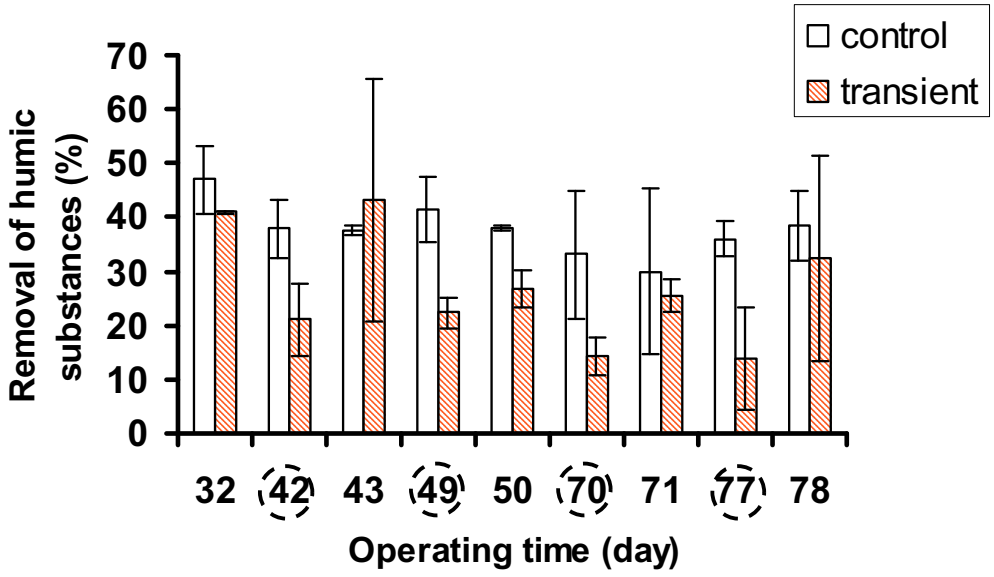


Figure 4.25 Removal of humic substances under DO transients: a decreasing removal efficiency of humic substance was shown in the transient SBRs ($p = 3.7 \times 10^{-4}$) on transient days 42, 49, 70 and 77 (as marked by cycles). The error bars are +/- one standard deviation of the measurements.

There were no substantial changes in the removal of proteins and polysaccharides upon DO variations. Variations of polysaccharides (bulk and bound) upon the DO transients were insignificant. With respect to proteins, the presence of high amounts of humic substances masked the concentrations of proteins. Thus, the modified Lowry method deployed in this work was not able to detect the levels of proteins in most cases. In regards to bulk proteins, there were undetectable levels in feeds and treated effluents except on transient days 70 and 126. On transient day 70, about 4 - 8 mg/L bulk proteins were detected in the treated effluents from the transient reactors (SBR3 and SBR4). On day 126, 13 mg/L bulk proteins were detected in the treated effluent from the transient reactor without adding extra chemicals (SBR4). Hence, the release of proteins under DO stress was detected occasionally, supporting the loss of EPS proteins due to the deflocculation. In contrast, throughout the experiments, approximately 10-

20 mg/L of bound EPS proteins were detected after being extracted from mixed liquor sludge using the CER method. However, no significant correlation was found between the oxygen variations and the changes in bound EPS proteins.

The DO variations had little impact on sludge settleability, as measured by SVI. From days 42 to 122, SVI in each SBR was monitored regularly and the numbers varied from 40 to 72 mg/g MLSS. There were no significant difference between control and transient samples upon the DO variations.

4.2.1.2 Levels of Extracellular Cations During DO Transients

Results showed a significant increase in bulk K^+ under DO limitation (Figure 4.26). Influents collected from the mill contained approximately 15 mg/L K^+, 60 mg/L Ca^{2+}, 10 mg/L Mg^{2+} and 300 mg/L Na^+. For normalization, the difference of cation concentrations between treated effluents and untreated influents were calculated. The bigger the difference, the more cations remained in the treated effluents. Under DO shortage (days 42, 49, 70 and 77), K^+ differences in the transient effluents were 2-8 times higher than those in the control effluents. This indicates that a significant amount of extracellular K^+ was released into the treated effluents under the DO stress. When the DO stress was removed, the observations on the following days (days 43, 50, 71 and 78) showed that the K^+ difference in the transient effluents dropped to the control levels, suggesting a correlation between DO transients and increasing K^+. The changes in bulk K^+ were consistent with the observations in batch experiments (see Section 4.1.1.3).

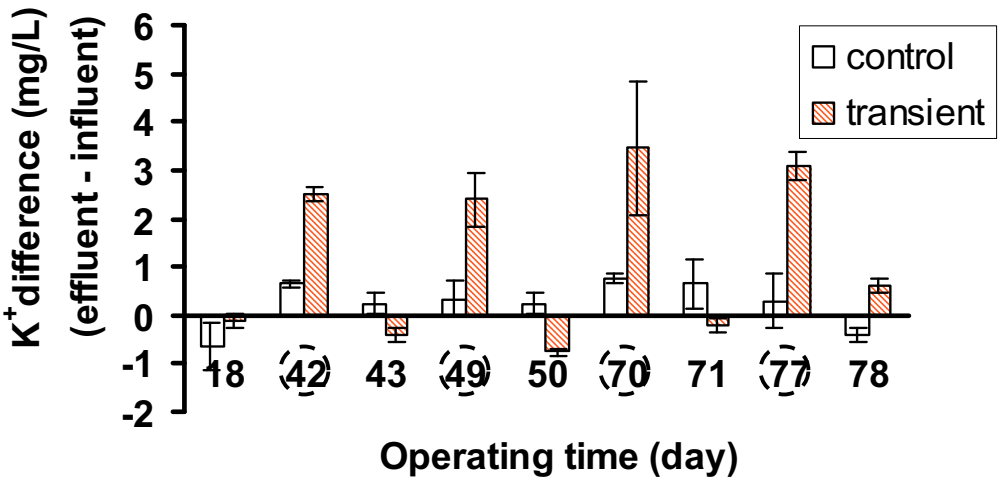


Figure 4.26 Changes in extracellular K^+ under DO transients: each column represents K^+ difference between the treated effluents and untreated influents. More K^+ were released into effluents on transient days 42, 49, 70 and 77 ($p = 2.1 \times 10^{-3}$). The error bars are +/- one standard deviation of the measurements.

In contrast to the changes in K^+, DO limitation induced a reduction of bulk Ca^{2+} by 1-3 times, as shown by the declining or negative values of Ca^{2+} difference on transient days (Figure 4.27). A reversible change in Ca^{2+} was observed when the DO level was restored. Compared to the changes in bulk K^+ and Ca^{2+}, no significant changes were found in extracellular Na^+ and Mg^{2+} (Appendix D). All the observations on bulk Ca^{2+}, Na^+ and Mg^{2+} at this stage were consistent with batch experimental results.

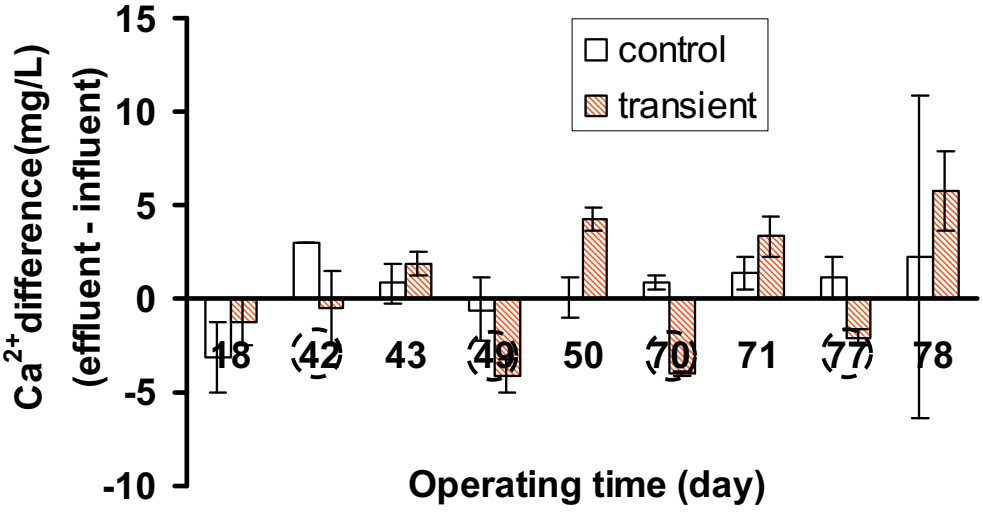


Figure 4.27 Changes in extracellular Ca^{2+} under DO transient: each column represents the Ca^{2+} difference between the treated effluents and untreated influents. O_2 stress caused a reduction of Ca^{2+} in treated effluents ($p=2.0 \times 10^{-3}$), as shown on transient days 42, 49, 70 and 77. The error bars are +/- one standard deviation of the measurements.

No conclusions were drawn from examining the levels of pellet-cations and total-cations in feeds and treated effluents, as well as the cations in mixed liquor. Regarding the pellet cations in treated effluents, though high levels of cations in the transient samples were obtained, this was attributed to the high SS concentrations under O_2 stress. When the cation levels were normalized to mg cations/g ESS, insignificant correlations were found between the DO variations and the cations in pellets. Similar to the previous batch results, cations in bound EPS were of much lower concentrations in comparison to bulk cations, and there was insignificant difference in the levels of bound cation between the control and transient samples.

4.2.2 Physical Properties of Bioflocs Under DO Variations

4.2.2.1 Particle Size Distribution

As shown in Figures 4.28 and 4.29, DO limitation generated more small particles (< 20 μm) in the treated effluents and mixed liquor. The effluents from control reactor SBR2 had approximately 30% of particles less than 20 μm, and 3% of particles below 12.5 μm (Figure 4.28). In contrast, the effluents from transient reactor SBR4 contained about 60% of particles less than 20 μm, and 8% of particles were below 12.5 μm (Figure 4.28). This was consistent with the observations on increasing SS and turbidity in the transient effluents. Similarly, there were approximately 35% of particles less than 20 μm in the transient mixed liquor whereas only around 15% of the particles with the same size were present in the control mixed liquor (Figure 4.29). These results implied that deflocculation under DO limitation originated from slough-off of big bioflocs, rather than from break-up of bioflocs, for the latter one would produce more medium-size particles.

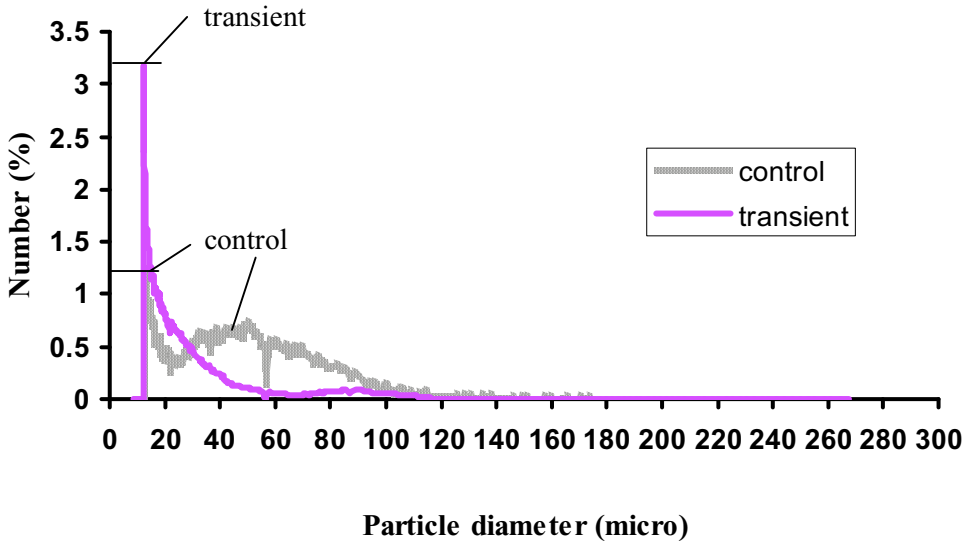


Figure 4.28 Effluent particle size distribution in treated effluents on day 98: The transient effluent had a higher amount of small particles (< 20 µm) than the control effluent.

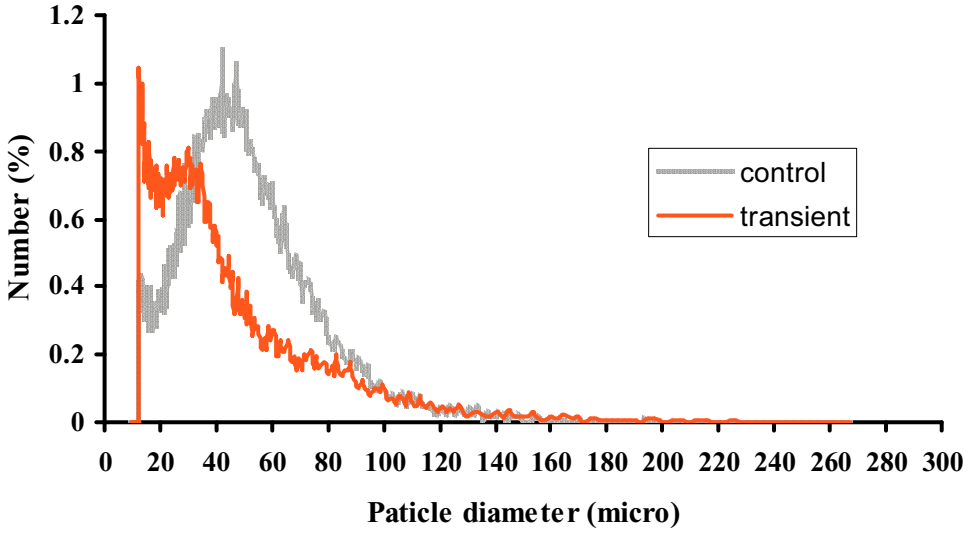


Figure 4.29 Particle size distribution of mixed liquor samples on day 98: The transient sample had a higher amount of small particles (< 20 µm) than the control sample.

4.2.2.2 Floc strength

Measurements on the shear strength of bioflocs showed that bioflocs exposed to a short-term low DO had a higher strength than control bioflocs. The impacts of DO variations on the floc strength were assessed by the changes in average shear strength. The average shear strength was measured as the shear stress at which 50% of tested bioflocs were broken. For the bioflocs between 45-63 μm, the average shear strength of the transient sample (SBR4) was higher than that of the control sample (SBR2) (Figure 4.30): The control sample had an average shear strength of 8 Pa whereas the transient sample had an average shear strength of 16 Pa. Similarly, for the bioflocs of 75-90 μm, the control sample had an average shear strength of 6 Pa whereas the transient sample had a value of 9 Pa. The higher floc strength after the deflocculation supported the model for bioflocs' structure: a strong inner layer surrounded by a weak outer layer (Section 2.3). The removal of loose particles from the outer layer by deflocculation left the strong inner layer behind and gave rise to an increasing strength of the remaining bioflocs.

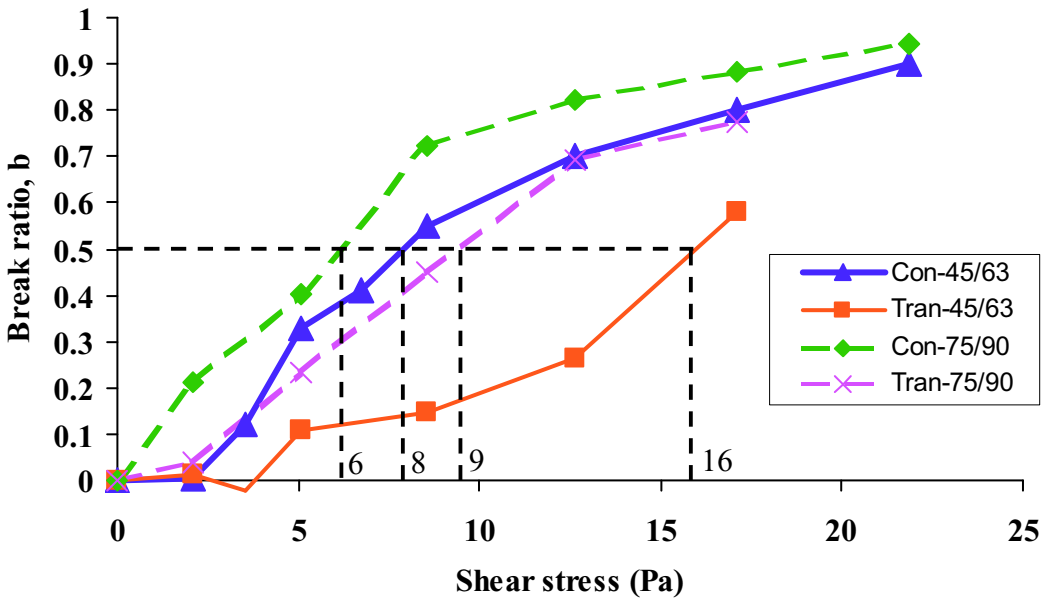


Figure 4.30 Shear strength of bioflocs from the control reactor (SBR2) and the transient reactor (SBR4): for both samples within 45-63 µm and 75-90 µm, bioflocs in SBR4 were stronger than those in SBR2.

4.2.3 Testing Strategies to Deter Deflocculation (Days 91-156)

The testing strategies concentrate on exploring an effective means to deter the deflocculation by alleviating the declining levels of extracellular Ca^{2+} or by preventing the release of K^{+} into the extracellular surrounding. This was implemented by sequentially adding $CaCl_2$, TEA chloride and glibenclamide into one control and one transient reactors for a period of 3 weeks, respectively. Effects of the added chemicals in minimizing deflocculation were shown by the difference in the performance of two transient reactors on the transient days.

4.2.3.1 Changes in Effluent Turbidity

Addition of extra Ca^{2+} partially alleviated the increment of turbidity under DO limitation (Figure 4.31). Effluent turbidity from the transient reactor with extra Ca^{2+} were significant

lower than those in the transient reactor without extra Ca^{2+}, as show by a reduction of 6%, 18%, 3% and 30% on transient days 91, 98, 105 and 112, respectively. However, addition of Ca^{2+} did not alleviate the ESS increments under the DO limitation, and there was insignificant difference between the transient reactors with and without extra Ca^{2+}.

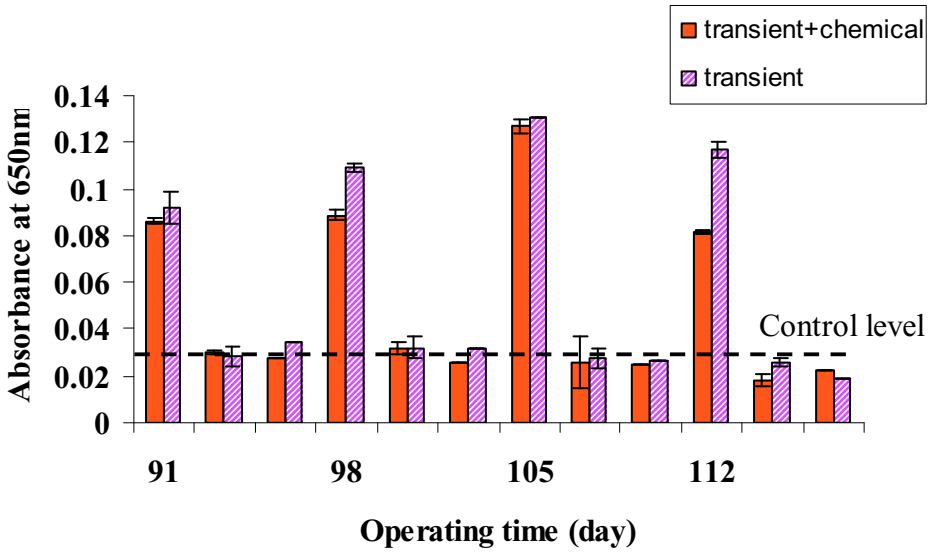


Figure 4.31 Changes of effluent turbidity upon DO variations from days 91 to 116. On transient days (labelled on x-axis), the addition of Ca^{2+} into the transient reactor partially alleviated the increasing turbidity, as shown by the lower turbidity than those in the transient reactor without Ca^{2+} ($p = 0.012$). The error bars are +/- one standard deviation.

4.2.3.2 Removal of SCOD

Over the period of days 91 to 156, there was no significant improvement in the removal efficiency of SCOD (Figure 4.32). A 35-60% decrease in the SCOD removal was sustained on the transient days, regardless of adding the selected chemicals.

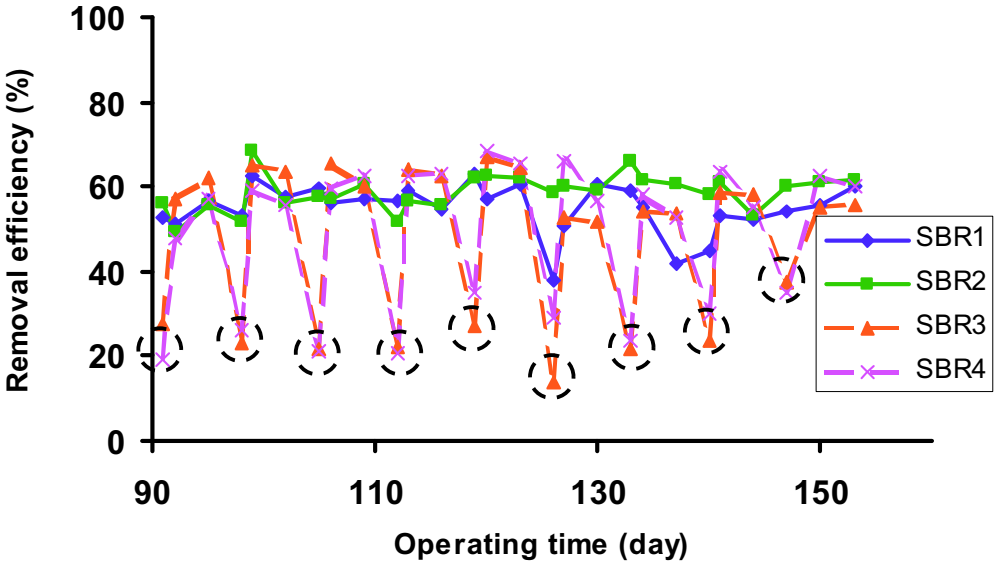


Figure 4.32 Removal of SCOD from days 91-156: In spite of the added chemicals, the removal of SCOD in the transient reactors declined under low DO (marked by cycles). Each data point represents the mean value with +/- one standard deviation.

4.2.3.3 Changes in Bulk Cations

From days 91 to 156, there were consistent increases in bulk K^+ and decreases in bulk Ca^{2+} under DO limitation in spite of adding the selected chemicals. The levels of bulk K^+ in the transient effluents continuously increased on the transient days. The levels of bulk Ca^{2+} in the transient effluents continuously declined on transient days except day 91 (Figure 4.34). All these demonstrated that the changes in extracellular K^+ and Ca^{2+} due to the DO limitation were not effectively adjusted by the addition of selected chemicals.

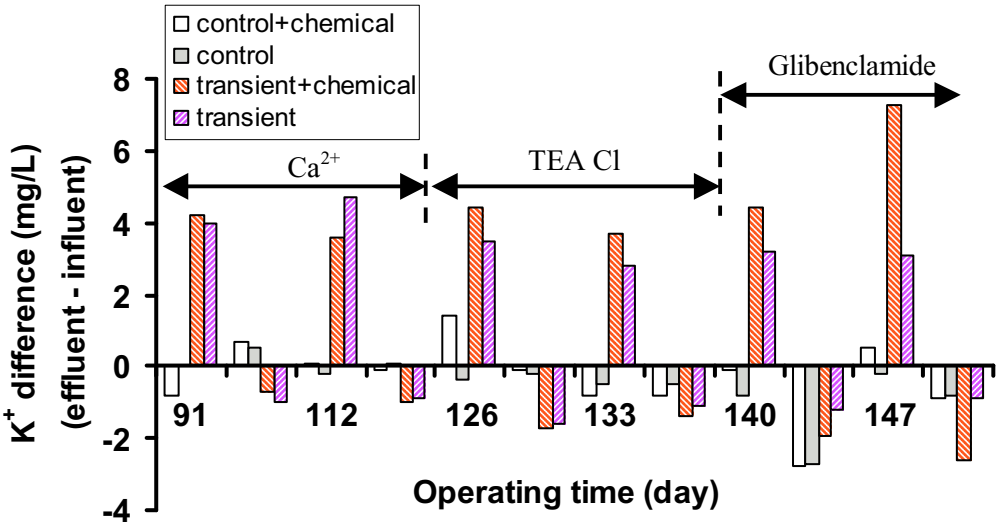


Figure 4.33 Changes in extracellular K^+ after adding the selected chemicals: adding extra Ca^{2+}, TEA chloride and glibenclamide failed to prevent the release of K^+ under DO limitation. Transient days are labelled on x-axis. The error bars are +/- one standard deviation.

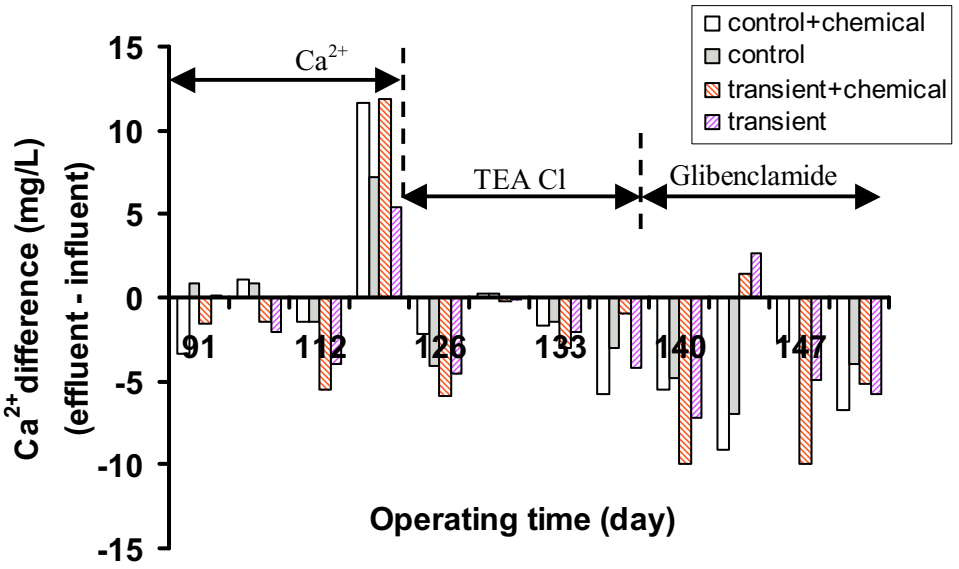


Figure 4.34 Changes in extracellular Ca^{2+} after adding the selected chemicals: adding extra Ca^{2+}, TEA chloride and glibenclamide failed to prevent the reduction of Ca^{2+} under DO limitation. Transient days are labelled on x-axis. The error bars are +/- standard deviation.

4.2.3.4 Outer Layer of Bioflocs

The addition of $CaCl_2$ reduced the average shear strength of bioflocs (Figure 4.35). For bioflocs within 45-63 µm, the average shear strength from the transient reactor enriched with Ca^{2+} (denoted by diamond) was over 10 Pa, higher than that of the control bioflocs (~8 Pa) but lower than that of transient bioflocs without extra Ca^{2+} (~16 Pa). The similar reduction in the shear strength was observed in the bioflocs with a size range of 75-90 µm.

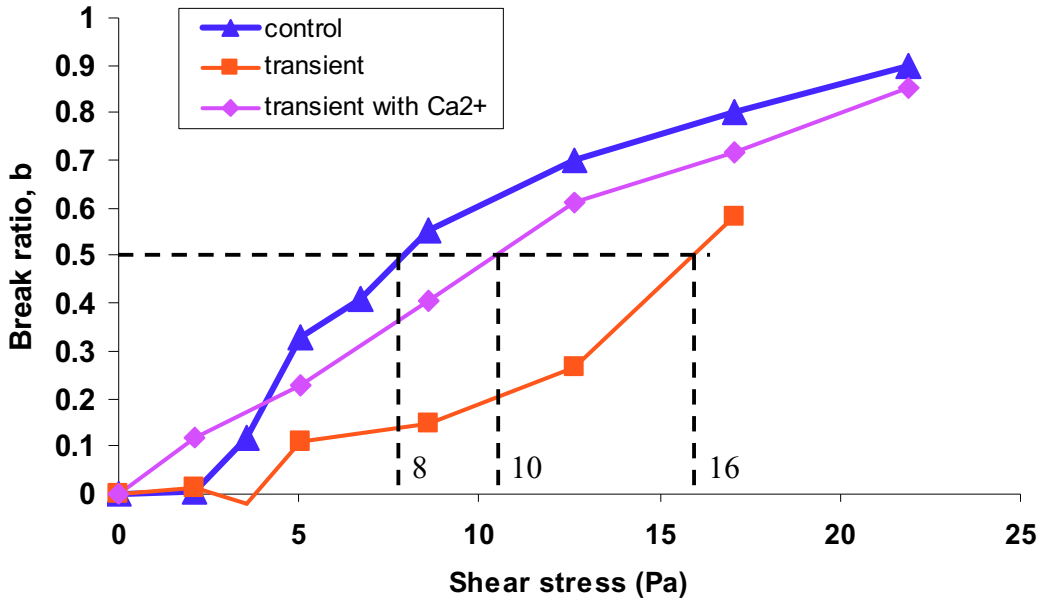
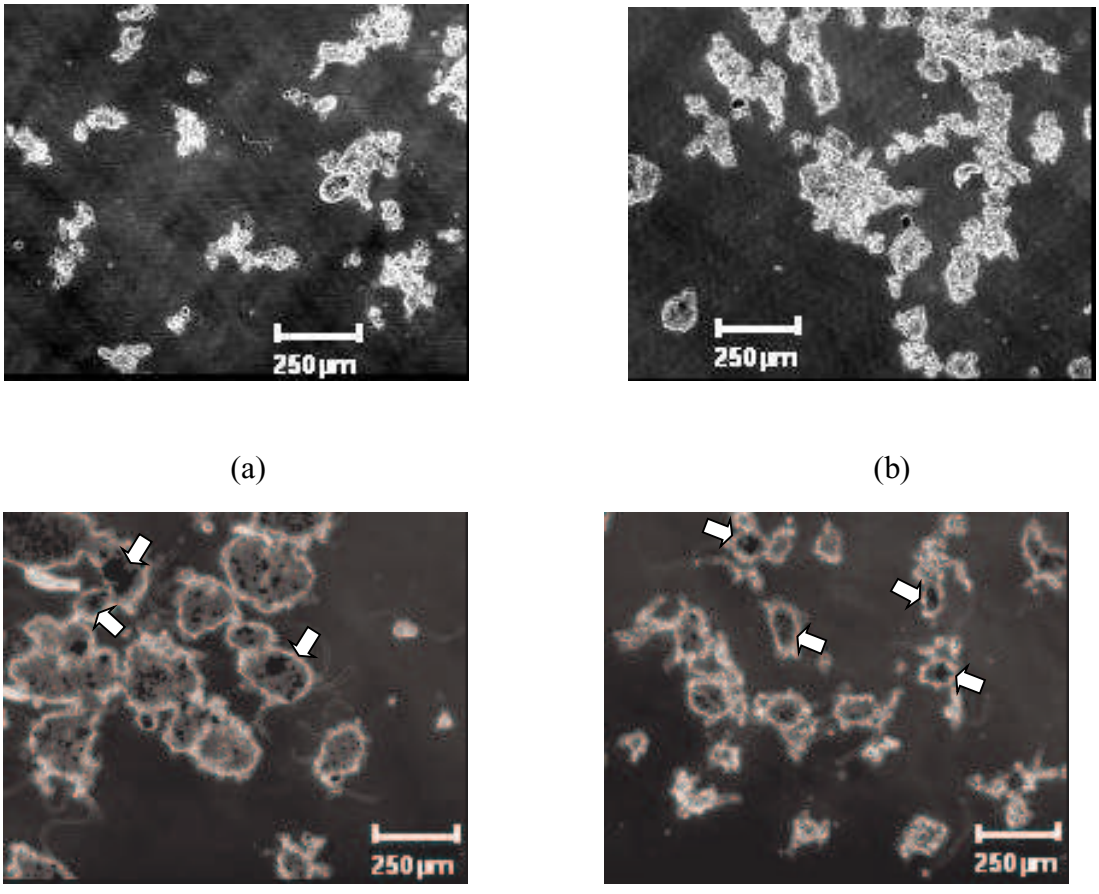


Figure 4.35 Distribution of shear strength of control reactor SBR2, transient reactors SBR3 (enriched with Ca^{2+}) and SBR4 for the bioflocs of 45-63 µm. Addition of Ca^{2+} reduced the shear strength of DO-stressed bioflocs.

4.2.3.5 Presence of Dense Cores

Microscopic images taken on day 133 showed a dense core present in the samples added by the selected chemicals (Figure 4.36). Compared to the samples without extra Ca^{2+} (Figures (a) and (b)), bioflocs added with the chemicals possessed a dense core (examples are denoted by

arrows) regardless of the DO stress (Figures (c) and (d)). Ca^{2+} was added into one control reactor (SBR 1) and one transient reactor (SBR3) from days 91 to 112, and TEA chloride was introduced into the reactors from day 116. The mixed liquor of activated sludge was not re-mixed and re-distributed before the introduction of TEA chloride. It was not clear whether the formation of dark cores was attributed to extra Ca^{2+} or TEA chloride or a combination of both. However, previous work has demonstrated the positive role of Ca^{2+} in floc stabilization (Higgins and Novak, 1997a-c; Biggs et al., 2001; Sobeck and Higgins, 2002). Therefore, it was reasonable to speculate that the presence of dense cores was related to the Ca^{2+} addition.



(a) (b)

(c) (d)

Figure 4.36 Microscopic observations of bioflocs on day 133: (a) Control (SBR2); (b) Transient (SBR4); (c) Control+ Ca^{2+}/TEA chloride(SBR1); (d) Transient + Ca^{2+}/TEA chloride

(SBR3). The images were taken by Axiovert 200 inverted microscope. The bar represents 250 μm.

4.2.3.6 Removal of Humic Substances

Adding $CaCl_2$ and glibenclamide improved the removal of humic substances in the transient reactor (Figure 4.37). On transient days (91, 98, 119, 126, 140 and 147), regardless of the added chemicals, the removal efficiency of humic substances in the control reactors was maintained around 40% and that in the transient reactors was below 30%. However, in comparing the difference between the transient reactors with and without the selected chemicals, addition of Ca^{2+} and glibenclamide enhanced the removal of humic substance under the DO limitation to some extent: On transient days 91 and 98, adding Ca^{2+} enhanced the removal of humic substances by approximately 45-50% (statistically significant with $p \approx 0.01$); on transient days 140 and 147, the presence of glibenclamide, in addition to $CaCl_2$, improved the removal of humic substances by 12-20%.

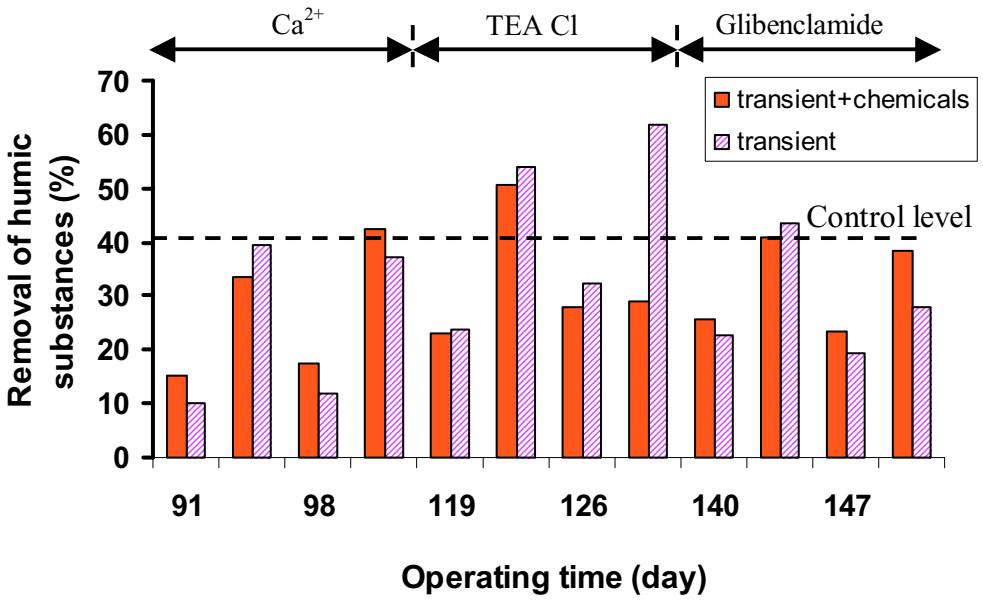
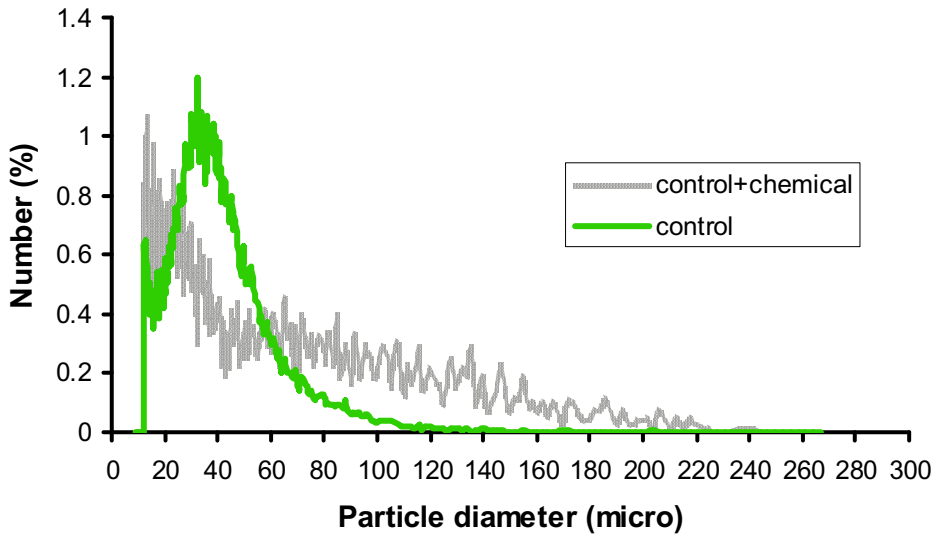


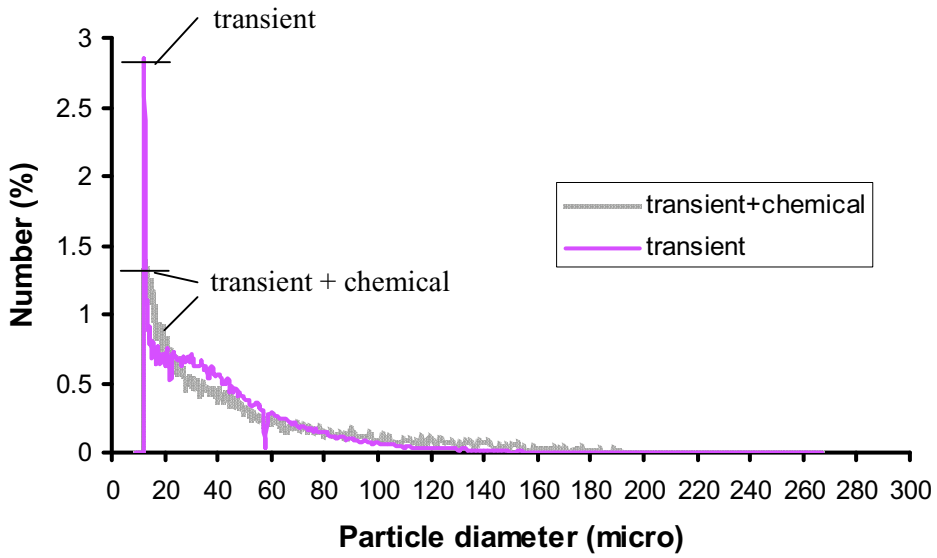
Figure 4.37 Removal of humic substances from days 91-150: Compared to the transient reactor without the added chemicals, the addition of Ca^{2+} and glibenclamide into the transient reactors appeared to enhance the removal efficiency on transient days 91, 98, 140 and 147, as shown by the higher columns than the ones without the chemicals.

4.2.3.7 Particle Size Distribution

Results from particle size distribution of mixed liquor at the end of the experiments showed that the addition of the selected chemicals reduced the number of fine particles and promoted the formation of bioflocs larger than 12.5 µm (Figure 4.38). Approximately 1% of particles less than 12.5 µm were present in the transient reactor added with the chemicals and around 0.6-0.8% of the particles below 12.5 µm were present in the control reactors. In contrast, around 2.8% of the particles within the same size range were present in the other transient reactor without the added chemicals. Thus, adding the chemicals reduced the number of fine particles under the DO limitation.



(a)



(b)

Figure 4.38 Particle size distribution of mixed liquor on day 153: (a): Control reactors; (b): Transient reactors. Addition of selected chemicals into the transient reactor decreased the number of small particles (< 12.5 μm) compared to the transient one without the chemicals.

4.2.4 Summary of Continuous Experiments

Overall, the impacts of short-term low DO have been demonstrated in a continuous system: There were significant increases in effluent turbidity, ESS, number of small particles, as well as decreases in the removal of SCOD and humic substances. The increases in bulk K^+ and bulk Ca^{2+} were correlated with sludge deflocculation.

Regarding the strategies for deterring deflocculation, addition of the selected chemicals did not completely stop the deflocculation under short-term low DO. There were improvements on reducing the effluent turbidity, enhancing the removal of humic substances, presence of a dense core and reducing the number of fine particles in treated effluents, compared to the DO-stressed samples without the added chemicals. However, compared to the control samples, deflocculation due to the DO limitation was sustained regardless of adding the chemicals.

4.3 Batch Tests on *E. coli*

To test the mechanism of deflocculation in a simple microbial system, batch experiments on *E. coli* suspensions exposed to DO limitation were carried out. The levels of extracellular K^+, Na^+, Ca^{2+} and Mg^{2+} were examined. Following discussion focuses on the changes of K^+ and Ca^{2+} only. The results on Na^+ and Mg^{2+} are presented in Appendix D.

4.3.1 Changes in Bulk Cations

Consistent with previous observations, levels of bulk K^+ significantly increased by 20 - 30% under O_2 limitation (Figure 4.39). The control and transient data were measured in independent duplicate samples (i.e., samples were taken from two independent flasks). The levels of bulk K^+ in the control samples were relatively stable over 90 min. In contrast, the

levels of bulk K^+ in the stressed samples increased by 16% after 30 min, and by over 30% after 90 min. This suggested that the increase in bulk K^+ was a stress response to O_2 limitation. Such increasing bulk K^+ under O_2 stress was consistently observed in separate experiments repeated on different days.

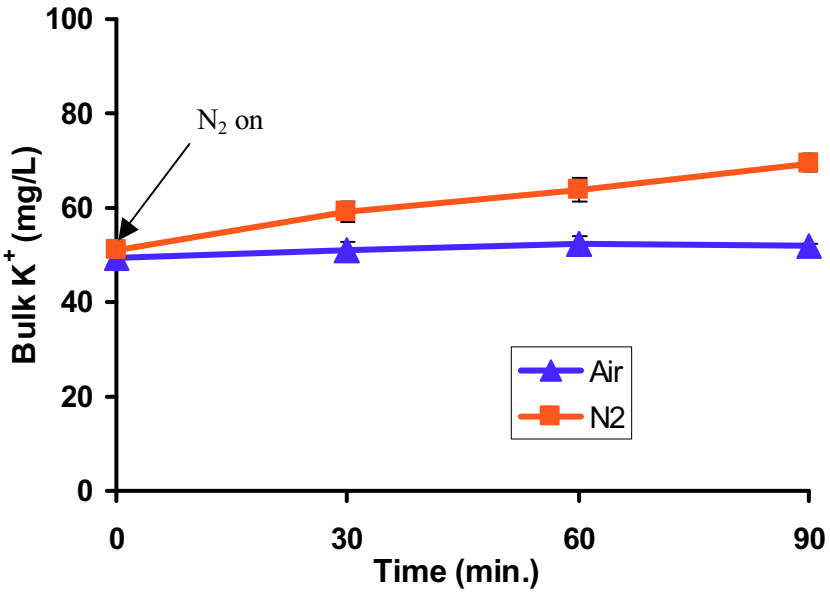


Figure 4.39 Changes of bulk K^+ in *E. coli* cultures responding to O_2 limitation: the stressed samples (denoted as "N2") had higher concentrations of bulk K^+ than the control samples ($p = 0.002$). The error bars are +/- one standard deviation of the measurements.

No conclusive observations were obtained for the changes in bulk Ca^{2+} under DO shortage. In a typical test, though bulk Ca^{2+} in the stressed samples decreased by over 20% after 60 min, the differences between the control data and stressed data were not statistically significant (Figure 4.40). In other repeated experiments, no significant decreases in bulk Ca^{2+} were observed over the experimental time.

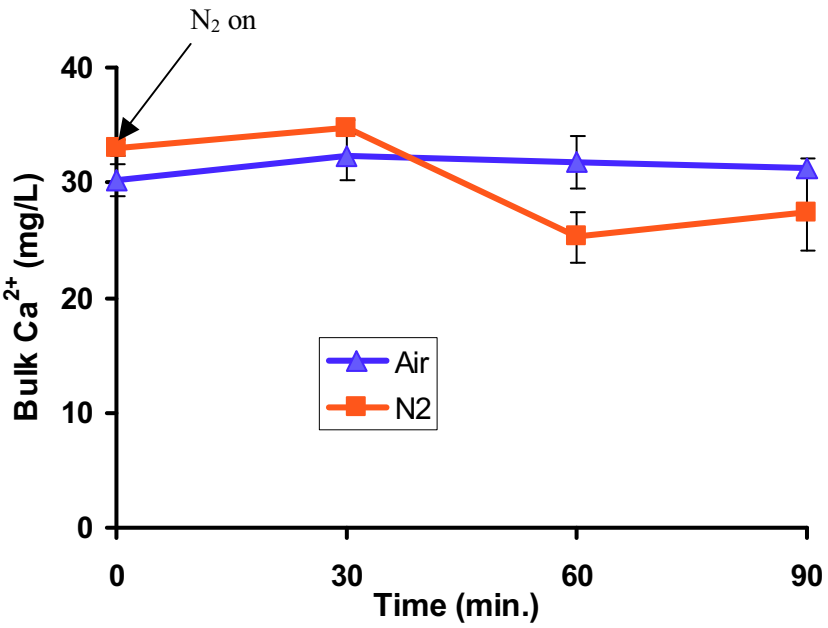
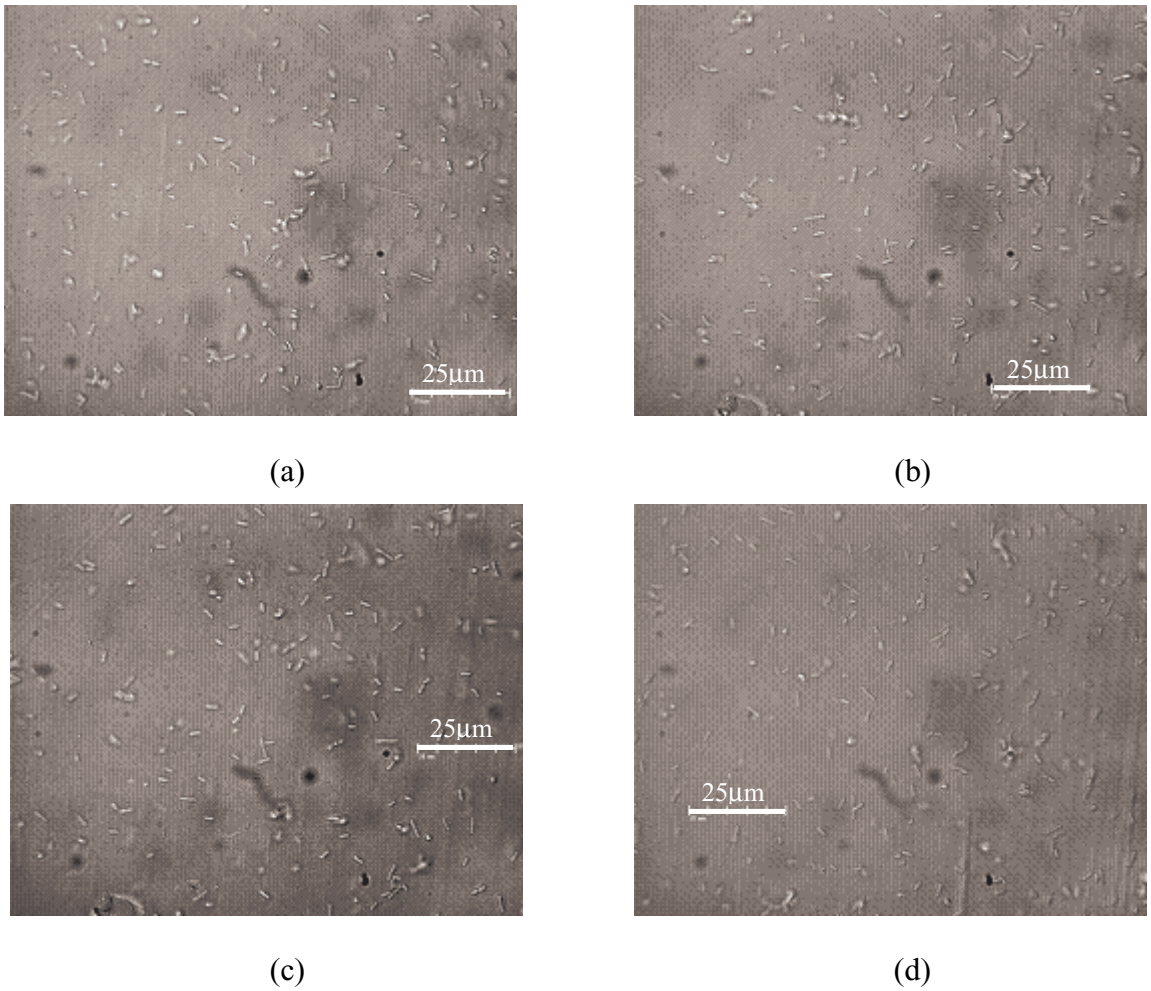


Figure 4.40 Changes of bulk Ca^{2+} in *E. coli* cultures responding to O_2 limitation: There were insignificant difference between the control and stressed samples ($p = 0.26$). The error bars are +/- one standard deviation of the measurements.

The aggregation of *E. coli* cells during the experimental period (≤ 120 min.) was not significant. The samples were observed under the microscope right before and after the experiments and no significant aggregation was observed (Figure 4.41). Thus, the increasing bulk K^+ was attributed to the release of cellular K^+ under the DO stress.



(a) (b)

(c) (d)

Figure 4.41 Microscopic observations of *E. coli* cells: (a) Control (t=0); (b) Transient (t=0); (c) Control (t=90 min.); (d) Transient (t=90 min.). The images were taken by Axiovert 200 inverted microscope. The bar represents 25 µm.

4.3.2 Addition of Valinomycin

Results from valinomycin tests showed insignificant improvement in the changes of bulk K^+ under DO deficit (Figure 4.42). Valinomycin is an antibiotic to promote the K^+ transport across the cell membrane. In this work, regardless of the presence of valinomycin (4.5-9 µM), the levels of bulk K^+ in the O_2-stressed samples were significantly higher than those in the control samples from 30 min to 120 min. There was no statistical difference between the O_2-stressed samples with and without valinomycin. This suggested that addition of valinomycin at

the current concentration range did not prevent the increasing levels of bulk K^+ under O_2 shortage.

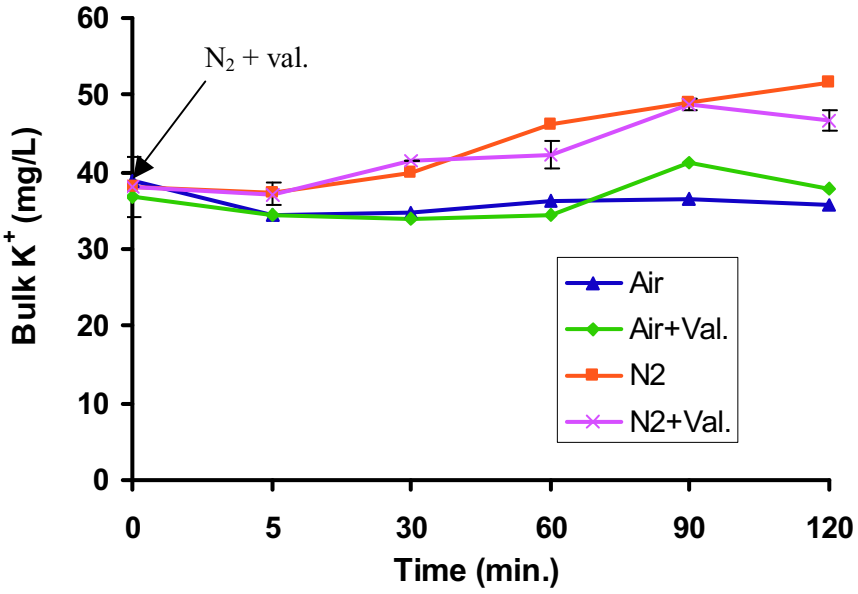


Figure 4.42 Changes in bulk K^+ with the addition of valinomycin: In spite of adding valinomycin, O_2 shortage caused increasing levels of bulk K^+. Valinomycin is denoted as "Val." The error bars are the +/- one standard deviation of bulk K^+.

4.3.3 Mass Balance of Cations

Apart from analyzing the cations in bulk solution, concentrations of ions in pellet and in total solution were analyzed. Theoretically, if the increasing bulk K^+ under the stress is from the release of cellular K^+, a similar magnitude of decreases in the amount of pellet K^+ should be observed, as well as insignificant changes in the amount of total K^+.

For K^+ levels in cell pellets, a decline by 20-35% over the time was observed in the O_2-stressed samples (Figure 4.43). The levels of pellet K^+ in the O_2-stress sample (denoted as "N2") declined from 4.6 mg/L to 2.8 mg/L within 120 min, and the levels of pellet K^+ in the

O_2-stress sample with valinomycin (denoted as "N2+Val.) declined from 4.7 mg/L to 3.6 mg/L within 120 min. This implied that the O_2-stressed cells released K^+ into extracellular environment. However, the amount of decreasing pellet K^+ was only approximately 20% of the amount of increasing bulk K^+. This may be explained by the incomplete cell lyses and experimental uncertainties in extracting pellet cations. A theoretical calculation showed that for the same volume of pellet cells, the concentrations of pellet K^+ should be around 35 mg/L if the cells were completely lysed and all the cellular K^+ were released. In this work, only 3-6 mg/L of pellet K^+ was detected in the experiments.

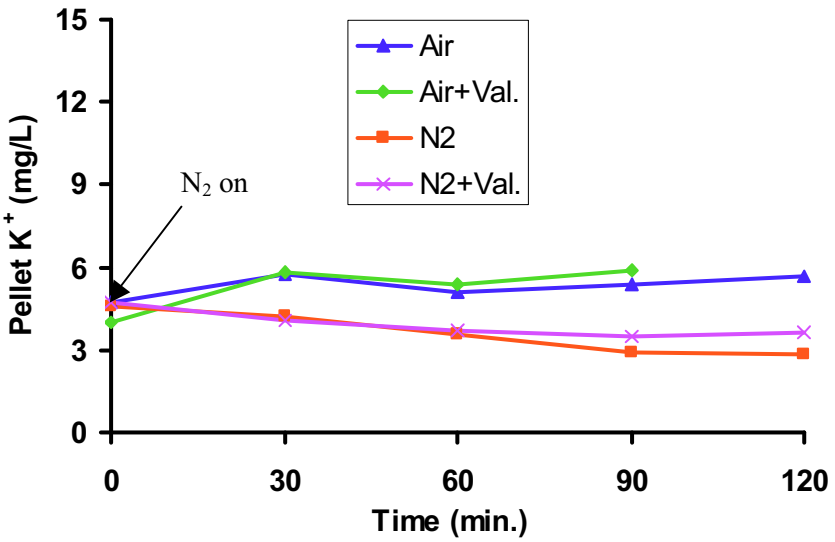


Figure 4.43 Changes of pellet K^+ in *E. coli* cultures. A decrease in the pellet levels of K^+ occurred in the DO-stressed samples.

An increasing level of total K^+ was observed in the O_2-stressed samples. Compared to the relatively stable levels in the control samples, the levels of total K^+ in the O_2-stressed samples slightly increased by 10% over the time. As discussed previously, the decreasing amount of

pellet K^+ did not completely account for the increasing amount of bulk K^+. Thus, the levels of total K^+ in the stressed samples increased over the time.

The increases in total K^+ was not due to the changes in cell density over the experimental time. A maximum 2-hour of aeration by air or N_2 appeared to have little impact on cell density. Viable cell counts showed that cell density varied from 10^9 to 10^{10} CFU/mL in different *E. coli* tests. There were insignificant difference of cell density between the control and stressed samples over the experimental time. However, it should be noted that viable cell counts were carried out by dilution of total 10^{-9}. Thus, any changes in cell density below 10^9 could not be detected.

4.3.4 Summary of *E. coli* Tests

E. coli experiments demonstrated that the increase in bulk K^+ was probably from the release of cellular K^+ and was a stress response to O_2 shortage. The increasing bulk K^+ under the O_2 limit was consistent with the previous findings from the experiments on activated sludge exposed to a short-term low DO.

CHAPTER 5 DISCUSSION

5.1 Deflocculation Under Transients of Short-term Low DO

5.1.1 Effects of Short-term Low DO on Activated Sludge

Overall, both batch and continuous experiments on activated sludge showed that transients of short-term low DO caused sludge deflocculation, as characterized by the changes in turbidity, ESS, number of small particles, and the release of soluble EPS components. A detailed discussion on each of them is presented as follows:

Both batch and continuous experiments demonstrated significant increases in effluent turbidity and ESS under the short-term low DO. Turbidity in the control samples remained relatively stable and low throughout the experimental time whereas turbidity in the DO-stressed samples increased by 20-fold in the batch experiments (Figure 4.1) and increased by at least 1-fold in the continuous experiments (Figure 4.23). Similarly, the concentrations of SS in the supernatant or treated effluents increased by 1-2 times under the short-term low DO in both batch and continuous systems (Figures 4.3 and 4.24). The increasing turbidity and ESS were attributed to more particles suspended in supernatant or treated effluents, indicating the occurrence of deflocculation under the short-term low DO. The changes in turbidity are consistent with the observations in previous studies on short-term DO disturbances (Starkey and Karr, 1984, Wilén and Balmér, 1998, 1999; Wilén et al., 2000a, b).

The parameters used to characterize deflocculation in this study, ESS and turbidity, each targets the particles with different sizes. According to the standard method for ESS measurement, the level of ESS included all the particles larger than 1.5 μm. In comparison, the

particles sizing from 0.45 μm to 1.5 μm were mainly counted in the absorbance readings at 650 nm as turbidity. Thus, a high ESS is not necessarily correlated with a turbid supernatant or a cloudy effluent. In the event of accidental washout of biomass (i.e., the pump accident on day 50 in the continuous experiments), large particles were carried over into treated effluents, leading to a significant increase in the level of ESS (Figure 4.24). But the effluent turbidity could remain within a low level if the amounts of fine particles (less than 1.5 μm) in the carry-over were minimal (Figure 4.23). Wilén and Balmér (1999) also commented that turbidity as absorbance reading at 650 nm mainly measured the particles smaller than 2 μm.

The occurrence of deflocculation under DO transients was also characterized by the increasing number of small particles (< 20 μm) in both mixed liquor and treated effluents (Figures 4.28 and 4.29). There was a correlation between the increasing small particles with sludge deflocculation. In particular, the number of particles less than 12.5 μm in the transient effluents was about 2 times higher than that in the control effluents (Figure 4.28). This implied that deflocculation under the DO transients was a process where small particles are removed from the bioflocs.

As a consequence of deflocculation, there were significant increases in soluble EPS proteins and humic substances under the short-term low DO. Batch experimental results showed a 20-fold increase in soluble proteins (Figure 4.5) and a 4-fold increase in soluble humic substances (Figure 4.7) under the DO limitation. Soluble EPS compounds were measured from the supernatant after a centrifugation at 10,000 g. There were no microbial cells observed in the supernatant. This suggested that the increases in soluble proteins and humic substances were mainly from EPS matrix rather than from inside the cells. The increases in soluble EPS

compounds have not been described in previous studies on short-term DO transients. Wilén et al. (2000b) examined soluble proteins, but did not observe significant changes under DO limitation.

Under the short-term low DO, reduction of overall SCOD removal could be likely related to the release of soluble EPS compounds, as well as a reduction in adsorption of colloidal particles. In the continuous experiments, the removal of SCOD was significantly decreased by at least 50% in the DO-transient reactors (Figure 4.21). The profile of SCOD within an 8-hour operating cycle showed a 10% increase of SCOD (~50 mg/L) in the transient effluents in the last 5 hours (Figure 4.22). This suggested that deflocculation due to the DO limit could trigger the release of EPS compounds, leading to more materials carried over into the treated effluents. If using an empirical formula $C_{16}H_{24}O_5N_4$ as a protein molecule (Sawyer et al., 1994), a preliminary estimation showed that a release of 30 mg/L soluble proteins (Figure 4.5) approximately contributed to extra 45 mg/L COD. Furthermore, the short-term low DO may affect the initial adsorption of colloidal particles onto bioflocs, as well as the release of some colloidal solids upon the occurrence of deflocculation. A batch study by Wilén and Balmér (1998) showed a reduction in the adsorption of colloidal solids under anaerobic conditions (DO = 0 mg/L), as compared to a high DO level (≥ 5 mg/L). Since transient DO levels in the present study dropped below 0.5 mg/L, a reducing adsorption capability of colloidal particles possibly occurred under the disturbance, thereby contributing to the reduction in the overall removal of SCOD.

5.1.2 Erosion-like Process of Deflocculation Under Short-term Low DO

5.1.2.1 Structure of Bioflocs

The experimental results on floc strength support the model of bioflocs' structure as a strong inner layer surrounded by a weak outer layer. Previous studies proposed that bioflocs contained a dense inner layer surrounded by a loose outer layer (Eriksson and Alm, 1991; Liao et al., 2002; Sheng et al., 2005). The findings from this work support the model of a two-layer structure, as shown by the increasing strength of remaining bioflocs after deflocculation (Figure 4.30). It is proposed that the outer layer of bioflocs is not as strong as the inner core. After removing the outer layer by deflocculation, the remaining inner core led to higher strength of bioflocs. A schematic representation of the change in bioflocs' structure due to deflocculation is presented in Figure 5.1.

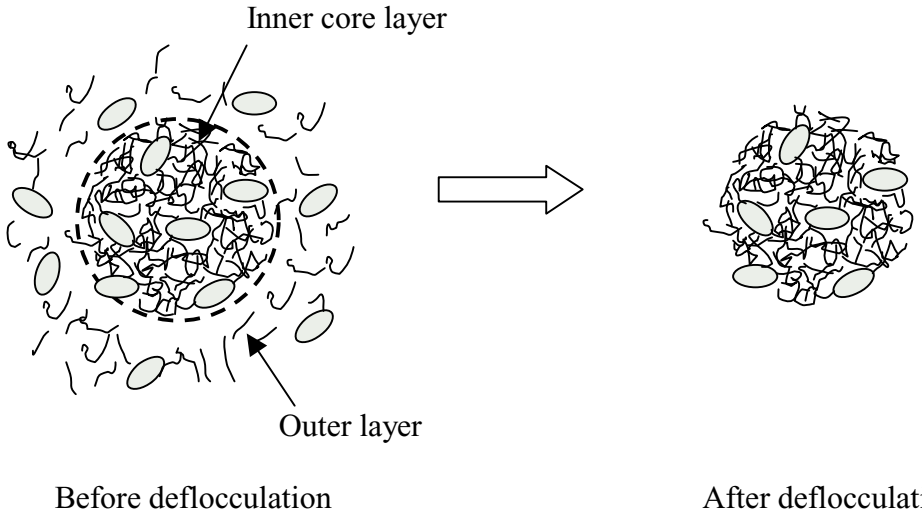


Figure 5.1 A schematic diagram of the change in bioflocs' structure under deflocculation (modified from Liao et al. (2002)).

It is suggested that the cells involved in short-term DO responses are mainly located in the outer layer of bioflocs. The outer-layer cells actively consume food and O_2, and are sensitive to the DO levels. In comparison, the inner-layer cells are acclimated to a relatively low DO level and are robust to the DO stress. Depending on the nature of bioflocs and sizes of bioflocs, thickness of the outer-layer varies from μm to mm determined by DO diffusion through bioflocs (Chiu et al., 2007; Daigger et al., 2007).

5.1.2.2 Deflocculation as an Erosion Process

The results of this study indicate that deflocculation due to short-term low DO is an erosion-like process by which surface particles on the outer layer of bioflocs are removed. This is supported by the changes in particle size distribution, turbidity and soluble EPS compounds along with the occurrence of deflocculation. As mentioned above, the structure of bioflocs has been recognized as a loose outer layer coated on a compact inner layer. This study showed a clear correlation between the increasing number of small particles (< 20 μm) and deflocculation (Figures 4.28 and 4.29). Typical sizes of bioflocs usually vary from 100-500 μm. The increasing number of particles less than 20 μm suggested that deflocculation was a process where the particles were removed from the outer layer rather than a process of bioflocs' break-up, for the latter one would tend to generate more particles with a larger size, e.g., above 50 μm. In terms of turbidity profile in batch experiments, the presence of a plateau suggested that after 4 hour of DO stress, the process of removing particles approached the inner compact layer (Figure 4.1). As a consequence, less particles were sloughed off and the changes in turbidity slowed down. In addition, a loss of the shell layer of bioflocs accounted for the increasing soluble proteins and humic substances under DO transients, for the soluble EPS compounds were mainly located in the shell layer (Figures 4.5 and 4.7). Collectively, all the evidence

suggests that deflocculation under short-term low DO is an erosion process of removing particles from the outer layer.

5.1.3 Deflocculation as a Process of Non-population Shift

5.1.3.1 Reflocculation upon the Resumption of DO Levels

Reflocculation occurred after DO limitation was removed, as shown by fast reversible changes in most parameters, including turbidity, SS, number of small particles, soluble proteins and humic substances, removal of SCOD and humic substances, and levels of extracellular cations. In the batch experiments, the reversible changes in turbidity and levels of bulk K^+ and Ca^{2+} were observed within 15 min of re-aeration (Figures 4.13 to 4.18). In the continuous experiments, following the DO transient cycles, measurements performed 24 hour later showed reversible changes in the above parameters. The occurrence of reflocculation within a short-time scale suggests that deflocculation under the short-term low DO is not a process of shifting in microbial species to adapt to the DO transients. Rather, it could be a physiological stress response at the metabolic level.

In general, a microbial stress response at a cellular level (metabolic or genetic) is much faster than that at a population level. A shift in microbial population would usually take days to weeks, depending on microbial species (MaCnaughton et al., 1999; Liu et al., 2000; Juang and Chiou, 2007). In comparison, a cellular stress response at the metabolic level or genetic level can be in an order of microseconds to minutes (Alon, 2007). Therefore, simply from the time scale of the reversible changes observed in this study, it is reasonable to suggest that a shift in microbial species is not a major stress response to short-term low DO.

5.1.3.2 Possible Explanations for Reflocculation

Both the growth of a new outer layer and cohesion of suspended particles could promote the reflocculation of activated sludge upon the restoration of DO. In the continuous experiments, unsettled particles detached from bioflocs were carried over into treated effluents and were discharged out of the system. Reflocculation observed in the measurements 24 hours later suggested the possible growth of a new shell layer upon the removal of DO limitation. Batch experimental results showed the reversible changes within 2 hours after the resumption of DO. Since small particles sloughed off from the remaining bioflocs were still suspended in the system, it was possible that after the resumption of DO, random adhesion of these small particles onto the remaining bioflocs facilitated the occurrence of reflocculation.

5.2 Mechanisms for Deflocculation

5.2.1 Changes in Extracellular K^+ and Ca^{2+} Under DO Transients

A correlation between sludge deflocculation and increasing bulk K^+ but decreasing bulk Ca^{2+} was consistently observed in both batch and continuous experiments. In the batch experiments, bulk K^+ increased by 40% and bulk Ca^{2+} decreased by 30% under the DO limitation (Figures 4.10 and 4.11). The ratio of bulk Ca^{2+} to K^+ decreased by 50%, from above 5 to 2.5. In the continuous experiment, the difference of bulk K^+ between treated effluents and untreated influents increased by at least 2-fold in the transient cycles (Figure 4.26). The difference of bulk Ca^{2+} decreased by 1-3 times in the transient cycles (Figure 4.27). The ratio of Ca^{2+} difference to K^+ difference decreased by 14%, from approximately 4.2 to 3.6. The increasing bulk K^+ has been observed in the deflocculation of activated sludge under the overloading of toxins (Bott and Love, 2002). In their batch experiments, extracellular soluble

K^+ nearly doubled after 5-hour exposure to a shockloading of N-ethylmaleimide. The changes in extracellular Ca^{2+} have not been described in other deflocculation studies. Overall, such changes in extracellular cations provide insight into the mechanism understanding of deflocculation.

In this work, the reduction of bulk Ca^{2+} under the DO limitation was not due to a connection between the bulk Ca^{2+} and the released soluble EPS compounds. Current results demonstrated insignificant difference of bulk Ca^{2+} before and after acidification of soluble EPS (Figure 4.12). Mg^{2+} can bind with EPS components as well. If the reduction in bulk Ca^{2+} were simply a re-connection with the EPS, a similar reduction in bulk Mg^{2+} would have been observed. However, in this work, there was no significant decline in bulk Mg^{2+}. Thus, it is reasonable to conclude that the re-connection between soluble EPS compounds and divalent cations is negligible.

The increasing level of bulk K^+ and the decreasing level of bulk Ca^{2+} observed in this work are possibly attributed to an efflux of cellular K^+ and an influx of extracellular Ca^{2+} across the cell membrane under the stress. For mammalian cells under an O_2 limitation, stressed cells can actively release cellular K^+ into extracellular solution but import extracellular Ca^{2+} into the cells (Haddad and Jiang, 1993; Jiang and Haddad, 1994). Though mammalian cells were not used in the present experiments, using the similar mechanisms of K^+ efflux and Ca^{2+} influx is feasible in interpreting the cation changes in an activated sludge system exposed to a DO limitation. Furthermore, despite the difference between eukaryotic and prokaryotic species, evidence has shown that there is a similarity with respect to the function and structure of specific K^+ channels present in both species (Mackinnon, 1998 and 2004).

Observations from E. *coli* tests support the notion that the increase in bulk K^+ is due to the release of cellular K^+ under O_2 limitation. Consistent with the findings in batch and continuous experiments on activated sludge, levels of extracellular K^+ in E. *coli* suspensions significantly increased under the O_2 deficit (Figure 4.39) and were prone to be reversible once the O_2 stress was removed. In *E. coli* suspensions, it was assumed that aggregation of the cells was not occurring and there was no presence of the biofloc matrix. Thus, the increasing level of bulk K^+ was simply due to the release of cellular K^+ and was a stress response to short-term O_2 shortage. Though *E. coli* is a facultative anaerobe and is able to initiate anaerobic pathway under O_2 limit, it is possible for the cells to possess different strategies for coping with O_2 stress, especially as an acute response occurred within seconds to minutes.

The release of K^+ and uptake of Ca^{2+} under the O_2 variations are different from the changes of K^+ and Mg^{2+} in a process of enhanced biological phosphorus removal (EBPR). K^+ and Mg^{2+} are the essential counterions of polyphosphates in living cells, due to their stable bonding with polyphosphates (Kortstee et al., 2000; Schönborn et al., 2001). In the EBPR process, orthophosphate is released in an anaerobic cycle through the degradation of intracellular polyphosphates. In the following aerobic cycle, extracellular phosphate molecules are taken up for generating energy and forming polyphosphates in cells. Simultaneously with the release and uptake of phosphates, K^+ and Mg^{2+} are released and taken up through the degradation and production of polyphosphates. The observations on the cation changes in this study are different from the EBPR process: Firstly, the EBPR process was not operated in this work; Secondly, there was no significant change in extracellular Mg^{2+} and the changes in the levels of K^+ and Ca^{2+} were in the opposite direction. Accordingly, the mechanisms for cation responses to short-term DO variations are different with cation changes in the EBPR process.

The magnitudes of changes in bulk K^+ and Ca^{2+} are correlated with the degree of deflocculation. Concentrations of bulk cations under temperature fluctuation from 30°C to 45°C were examined as an undergraduate thesis project (Chmiel, 2006). Compared to the temperature disturbance, the short-term low DO imposed a much higher degree of deflocculation in the first 2 hours, as characterized by the 6-fold higher turbidity (Appendix G). Concurrently, in the first 2 hours, the increasing amount of bulk K^+ under the low DO was 6-fold higher than that under the temperature upshift. Similarly, the decreasing amount of bulk Ca^{2+} under the low DO was up to 28-fold compared to that under the temperature changes (Appendix G). Thus, the magnitude of turbidity changes is correlated with the magnitude of cation changes under the disturbances.

Reversible changes in the levels of bulk K^+ and Ca^{2+} are probably attributed to an active adaptation of cells in response to the resumption of DO. In the batch experiments, once the DO stress was removed, there was no continuous release of cellular K^+. Instead, the cells could actively take up extracellular K^+ for their metabolism. Thus, the levels of bulk K^+ declined and eventually returned to control levels. In the continuous experiments, apart from no release of cellular K^+, the extra K^+ in the system was washed out into treated effluents. All of these lead to the reversible changes in bulk K^+ upon the DO variations. Similarly, in response to the restoration of DO levels, the cells might release extra Ca^{2+} taken under the stress, so as to return to an "unstressed" condition. Under a normal condition, cells always extrude Ca^{2+} and maintain a low intracellular Ca^{2+} level compared to extracellular surrounding (Lynn and Rosen, 1987). Thus, an increasing level of bulk Ca^{2+} occurred once the DO stress was removed.

5.2.2 Proposed Mechanism for Deflocculation Under the Short-term Low DO

In this study, it is proposed that deflocculation can be a protective response of perturbed cells to an undesirable living environment. Aerotaxis is a bacterial response to an oxygen gradient by moving either toward or away from oxygen, and is commonly present in bacterial cells (Taylor et al., 1999; Madigan et al., 2000). In activated sludge systems, cells tend to aggregate as bioflocs where various microbial cells develop an effective interaction and synergistic relationship within the aggregates. However, when the aerobic cells (i.e., the cells located on the outer layer of bioflocs) sense a signal of a low oxygen concentration, it is possible that the cells exhibit an aerotaxis behaviour, in an attempt to move away from the hypoxic condition. To achieve this, one of the effective means could be to activate fluxes of specific cations, e.g., K^+ moving out and Ca^{2+} moving into the cells. Upon the reducing number of available binding sites (e.g., Ca^{2+}) in the extracellular matrix, deflocculation is prone to occur, allowing the cells to move away from the stressed surrounding.

There is evidence that the decreasing ratio of bulk Ca^{2+} to K^+ is the cause for deflocculation under the DO limitation. In extracellular environment, more K^+ and less Ca^{2+} under the DO limit result in less available binding sites for a stable aggregation of bioflocs. Given the important role of divalent cations in bioflocs' flocculation (Higgins and Novak, 1997a-c; Biggs et al., 2001; Sobeck and Higgins, 2002), it is proposed that the decreasing ratio of extracellular Ca^{2+} to K^+ causes sludge deflocculation under the short-term low DO. If deflocculation were the trigger, it would be feasible to observe increasing levels of all the cations embedded in EPS matrix, including K^+, Ca^{2+}, Mg^{2+} and Na^+. However, in this work, there were significant decreases in bulk Ca^{2+} and insignificant changes in Na^+ and Mg^{2+} under the deflocculation. The release of cellular K^+ through a glutathione-gated K^+ efflux channel has been proposed as the

mechanism for deflocculation under the overloading of toxins (Bott and Love, 2002, 2004). All of these findings indicate that deflocculation of activated sludge under environmental disturbances can be caused by the changes of ionic strength in extracellular solution.

Results from this work suggest that effects of externally adding K^+ onto bioflocs are different from the effects of releasing cellular K^+ into biofloc matrix. Batch experiments in this work showed that simply adding K^+ into activated sludge, at a final concentration of approximately 0.3 mM, did not cause deflocculation (Figure 4.13). Bruus et al. (1992) observed that a concentration of at least 100 mM of extracellular K^+ caused deterioration of floc stability, as shown by an increase in turbidity. Consistently, in the sample treatment of one batch test in this study, due to an accidental miscalculation, 40 mL of K^+ at a concentration of 100 mM was introduced into a sludge pellet and caused much cloudy supernatant and high turbidity (absorbance at 650 nm was around 0.12). Thus, the external addition of 0.3 mM of K^+ in this work was far below the level known for causing deflocculation. Different from the K^+ addition, the release of cellular K^+ could impose a high concentration of local K^+ in the cell's vicinity, since the aqueous volume outside cells but within bioflocs is much smaller than that of bulk solution. In other words, the magnitudes of K^+ levels around the DO-stressed cells can be much higher than those observed in the bulk solution.

To further explain a possible high-level of K^+ around the cells, a simplified calculation was carried out based on the batch results: In the batch experiments, 30 mL of mixed liquor sample had approximately 0.072 g dry solids if average MLSS was 2400 mg dry solids/(litre of mixed liquor). If 70 wt% of dry solids were microbial cells (based on the ratio of MLVSS/MLSS over the experimental period), and it was assumed that each cell had a volume of 10^{-15} litres and

weighed 10^{-12} g (based on an *E. coli* cell), then the cell volume in 30 mL of mixed liquor would be 0.0504 mL. It was assumed that local aqueous volume around cell vicinity was half of the cell volume (Bott and Love, 2004). Thus, in 30 mL mixed liquor, the local aqueous volume would be 0.0252 mL and the total volume of bioflocs would be 0.076 mL. It was assumed that the volume of bioflocs was identical to the volume of sludge pellet after the centrifugation. Accordingly, the volume of the supernatant was approximately 29.92 mL. In average, an extra 5 mg/(litre of supernatant) of bulk K^+ was observed under the DO stress from a 30 mL of mixed liquor sample after the centrifugation. If all the extra bulk K^+ was from the cellular release, then a release of cellular K^+ would impose a local K^+ concentration of approximately 5936 mg K^+/(litre of local aqueous volume), i.e, 152 mM of K^+ around the cell's vicinity. Such a local K^+ level was in the same magnitude as 100 mM known to cause floc deterioration. Similarly, Bott and Love (2004) estimated the release of cellular K^+ under toxic disturbances generated approximately 320 mM of local K^+ within bioflocs. Thus, an efflux of cellular K^+ can impose a high level of K^+ in the cell's vicinity, thereby reducing the local ionic strength and causing sludge deflocculation.

Deflocculation under short-term low DO was not caused by cell lyses. The amount of cell lyses due to the O_2 stress were insignificant in this work, as shown by reversibility in the most measurable parameters within minutes to hours (e.g., turbidity, ESS, soluble proteins and humic substances, extracellular cations). In addition, cells contain different ions, including K^+, Na^+, Ca^{2+} and Mg^{2+}. In particular, cellular levels of K^+ and Mg^{2+} are higher than those in extracellular solution. Thus, if cell lyses were significant under short-term low DO, increases in all cellular cations would be observed. Rather, in this work, increases in K^+ but decreases in

Ca^{2+}, and insignificant changes of Na^{+} and Mg^{2+} in extracellular solution were observed under the low DO. Hence, cell lyses under short-term DO limitation are minimal.

It is proposed that deflocculation due to the ionic fluxes under the DO limit is a physiological response at the metabolic level. Under the DO transients, specific membrane-bound ion transporters are activated, allowing K^{+} and Ca^{2+} to move across the cell membrane. Evidence shows that in mammalian cells, an activation of a specific voltage-gated Ca^{2+} channel can only take less than 450 microseconds (Marrion and Tavalin, 1998). Jiang and Haddad (1994) also reported that the changes in membrane potential and ionic responses occurred within 1 min in the neuronal cells of rats. Alon (2007) summarized typical time scales of *E. coli* (a prokaryotic species), *S. cerevisae* (a yeast cell) and a human fibroblast cell (a mammalian cell) at different cellular levels: A metabolic response is rapid and often is in a scale of microseconds. There is not much difference among *E. coli*, yeast and mammalian cells for metabolic-level responses. For example, a switch between an active state to an inactive state of proteins only takes around 1-100 microseconds in *E. coli*, yeast and mammalian cells. In comparison to the metabolic responses, genetic responses are relatively slow (i.e., in minutes). It takes longer for mammalian cells to respond at the genetic level: Time to transcribe a gene takes around 1 min in *E. coli* and yeast cells, and takes around 30 min in mammalian cells; time for gene translation takes around 2 min in *E. coli* cells, and takes around 30 min in the mammalian cells. Thus, based on the time scale, it is surmised that deflocculation of activated sludge, as a stress response to the DO limit, could be a physiological response at the metabolic level.

Although Fe^{3+} can be an important ion in holding flocs together, it was not considered as a significant factor in this study because of the morphological change of deflocculation under DO limitation, the type of organisms involved in the DO stress, and the low amount of Fe expected in the system. Firstly, removal of Fe was proposed to generate a deflocculation as a process of bioflocs' breakup (Keiding and Nielsen, 1997; Nielsen and Keiding, 1998) rather than an erosion process observed in this study (Section 5.1.2.2). Previous studies suggested that most of the Fe present in bioflocs was tightly bound with EPS compounds or entrapped in the flocs as an inorganic precipitate (e.g., $Fe(OH)_3$), and the reduction of Fe^{3+} occurred in the inner bioflocs (Rasmussen and Nielsen, 1996, Keiding and Nielsen, 1997). Accordingly, removal of Fe^{3+} was expected to trigger deflocculation of sludge as a dissolution-like process (Keiding and Nielsen, 1997; Nielsen and Keiding, 1998). Secondly, different organisms are involved in the deflocculation by reduction of Fe^{3+} and the deflocculation by short-term low DO. Specific anaerobic organisms, i.e., iron-reducing bacteria and sulphate-reducing bacteria, are responsible for biological or indirect chemical removal of Fe^{3+}. The anaerobic bacteria tend to locate inside the bioflocs where the DO level is low (Gerardi, 2002; Nielsen et al., 2002). In comparison, it is mainly the cells located in the outer layer of bioflocs that are involved in the stress response to short-term DO limitation (Section 5.1.2.1). Organisms in the shell layer are dominated by actively growing aerobic cells, which are different from the anaerobic organisms accommodated inside the bioflocs. Thirdly, as aforementioned (Sections 2.3 and 3.2.3), based on the past research (Bott and Love, 2002; Morgan-Sagastume, 2003), the amount of Fe present in the fresh mixed liquor activated sludge was estimated to be low (less than 4 mg Fe/g MLVSS). Evidence shows that with such a low level, the role of Fe in deflocculation of sludge would be minor (Park et al., 2006b). Therefore, it is likely that Fe is not a significant

mechanism for the deflocculation under short-term low DO. However, there would be value in measuring Fe in future studies to completely rule out the significance of Fe in the deflocculation under the short-term DO disturbance.

Other possible mechanisms for deflocculation should not be eliminated. Activated sludge is a mixed community of various microorganisms. Different cells may have different surviving strategies against disturbances. Proteins are often the first inducible components to a stress response. The activation of extracellular enzymes to degrade EPS components has been proposed as the mechanism for biofilm detachment (Boyd and Chakrabarty, 1994; Lee et al., 1996; Allison et al., 1998; O'Toole et al., 2000; Stoodley et al., 2002). The enzymatic tests conducted in this work highlight the importance of EPS proteins in preventing sludge deflocculation. Thus, it is also possible that short-term low DO triggers an activation of specific enzymes to degrade EPS proteins, thereby causing deflocculation.

5.3 Strategies for Deterring Deflocculation

5.3.1 Improvements on Treatment Performance and Properties of Bioflocs

In spite of incomplete success in minimizing deflocculation under short-term low DO, treatment performance was partially improved by adding selected chemicals, especially Ca^{2+}. Adding Ca^{2+} promoted the formation of relatively large biofloc fragments and reduced the carry-over of fine particles (< 1.5 μm) during deflocculation. The surface of bioflocs is normally negatively charged, the presence of Ca^{2+} can enhance the adhesion of small particles onto the bioflocs by a bridging effect. Hence, a portion of particles less than 1.5 μm was captured by extra Ca^{2+}, alleviating the turbidity increments under the DO transients (Figure

4.31). As well, the addition of Ca^{2+} to some extent prevented the loss of weak outer layer during deflocculation, as shown by a reduction in the overall floc strength (Figure 4.35).

5.3.2 Possible Explanations for Unsuccessful Strategy Tests

The unsuccessful attempt in stopping deflocculation by extra Ca^{2+} is attributed to the presence of diffusion barrier and the loss of soluble EPS as available binding sites. The presence of diffusion barrier from biofloc matrix minimizes the effectiveness of strategies explored in this work. Stewart (1998) summarized that depending on the nature of biofilm and the size of solutes, diffusion of ions and organic compounds in biofilm varies from 15% to 50% of that in pure water. There should be a similar magnitude of reduction in the diffusivity of ions and organic compounds in bioflocs. Due to the presence of a diffusion barrier and small aqueous volume entrapped within the bioflocs, the levels of increasing K^{+} and decreasing Ca^{2+} locally (i.e., around the vicinity of the stressed-cells) under the DO limitation were far higher than the bulk values observed in the experiments. Simply adding Ca^{2+} in an equal amount to the reduction of bulk Ca^{2+} was insufficient and did not stop the deflocculation. In addition, the bond between negative functional groups on EPS and extracellular Ca^{2+} is one of the dominant forces to hold bioflocs together. The loss of soluble EPS components (e.g., proteins) under the DO stress can reduce the number of available binding sites. All of these account for the unsuccessful attempt in the Ca^{2+} strategy.

Apart from the diffusion barrier in EPS, the outer membrane of gram-negative cells, as well as cell walls, provides another hindrance for diffusion of TEA chloride, glibenclamide or valinomycin. The outer membrane of gram-negative cells is difficult for the passage of organic compounds, especially for a compound with a high molecular weight (Ahmed and Booth, 1983;

Walderhaug et al., 1987). Thus, the transportation or diffusion of TEA chloride, glibenclamide or valinomycin may be limited by the outer-membrane of gram-negative species. The organic chemicals are unable to reach the ion transporters embedded in cytoplasmic-membrane. This may account for the ineffectiveness of TEA chloride, glibenclamide and valinomycin in deterring deflocculation. In studying the effect of valinomycin on uptake of K^+ in *E. coli* cells, researchers either used *E. coli* vesicles (Bhattacharyya et al., 1971) or *E. coli* mutants which had no permeability barrier to valinomycin (Ahmed and Booth, 1983).

CHAPTER 6 OVERALL CONCLUSIONS

The focus of this Ph.D. research is to enhance the fundamental understanding of deflocculation of bioflocs under transients of short-term low DO concentrations. Deflocculation of bioflocs was demonstrated in a batch system using municipal activated sludge, as well as a continuous system treating wastewater from a pulp and paper mill. A mechanism for deflocculation was proposed. Batch results on *E. coli* cells responding to O_2 stress support the fundamental understanding of deflocculation. The main conclusions arising from this study are as follows:

1. **Transients of short-term low DO cause deflocculation of bioflocs in activated sludge.** The deflocculation is characterized by:

    - Significant increases in effluent turbidity (by 20 times in the batch tests and by 1-2 times in the continuous tests);
    - Significant increases in the concentrations of suspended solids in supernatant or treated effluents (by 1-2 times);
    - Increasing numbers of small particles (< 12.5 μm) in treated effluents (by 2 times);
    - Significant increases in soluble proteins (by 20 times) and soluble humic substances (by 4 times);

2. **The DO limitation causes a significant increase in extracellular K^+ and a substantial decrease in extracellular Ca^{2+} and insignificant changes in extracellular Na^+ and Mg^{2+}.** In the batch tests, extracellular K^+ increased by 40% whereas the

extracellular Ca^{2+} decreased by 30% under the DO limitation. In the continuous experiments, the difference of bulk K^+ between treated effluents and untreated influents increased by more than 2 times under the DO limitation. In contrast, the difference of extracellular Ca^{2+} decreased by 1-3 times under the DO limitation.

3. **It is proposed that the decreasing ratio of extracellular Ca^{2+} to K^+ is the cause for deflocculation of bioflocs under the transients of short-term low DO.** The short-term low DO triggers the fluxes of K^+ and Ca^{2+} across the cell membrane, as an efflux of cellular K^+ but an influx of extracellular Ca^{2+}. This gives rise to a decreasing ratio of extracellular Ca^{2+} to K^+, thereby causing sludge deflocculation. E. *coli* tests support that the O_2 shortage induces the release of cellular K^+ into extracellular environment and the increase in extracellular K^+ is a stress response to O_2 limitation.

4. **Deflocculation of activated sludge under the DO limitation is proposed to be an erosion-like process by which particles are mainly removed from the outer-layer of bioflocs.** This is supported by increasing number of small particles, increasing floc shear strength of the remaining bioflocs after deflocculation, the presence of a plateau in the turbidity profile of DO-stressed samples, and increasing concentrations of soluble EPS proteins and humic substances under the DO limitation.

5. **The addition of Ca^{2+} partially detains the loss of outer-layer under the DO limitation, as shown by decreases in effluent turbidity and floc strength compared to the DO-stressed bioflocs without extra Ca^{2+}.**

6. **Addition of selected chemicals ($CaCl_2$, TEA chloride and glibenclamide) partially improves the treatment performance under the DO disturbances.** This is shown by

the decreases in turbidity (5-30%) and the amounts of humic substance in the transient treated effluents (by 30-50%), the formation of a dense inner layer, and a decrease in the number of small particles (< 12.5 μm) in comparison to the DO-stressed samples without the selected chemicals.

7. **Evidence supports that deflocculation of bioflocs under the transients of short-term low DO is a physiological response at the metabolic level.** Reflocculation of activated sludge occurred once the DO stress was removed, as demonstrated by reversible changes in the measurable parameters (items 1-2) within minutes to hours. This suggests that the deflocculation under the short-term low DO is not a process of selecting robust microbial species to the DO stress. Instead, it could be a stress response at the metabolic level.

8. **Results from enzymatic tests show that compared to polysaccharides, proteins are more important in preventing deflocculation of bioflocs.**

CHAPTER 7 ENGINEERING AND SCIENTIFIC SIGNIFICANCE

This work is first to examine the impacts of short-term low DO in a continuous system using industrial wastewater. Currently, published studies on the DO impacts are mainly in batch systems. Only one study examined the changes in turbidity of treated effluents in a continuous system fed with synthetic feeds (Starkey and Karr, 1984). In this research, the impacts of short-term transients of DO concentrations were investigated in a continuous system treated with primary treated wastewater from a bleached kraft pulp and paper mill for over 5 months. The DO impacts were demonstrated, as shown by the changes in turbidity, ESS, EPS components, removal of SCOD, particle size distribution, and floc strength. Results of this work present a detailed picture of the impacts of short-term low DO on treatment plants and provide a valuable information to treatment plant operators.

This work is first to measure the changes in extracellular cations under the transients of short-term low DO. To our knowledge, no studies are available on examining the levels of extracellular cations in response to O_2 limit in activated sludge systems. In this work, concentrations of extracellular cations under the DO variations were examined in both batch and continuous systems, and consistent findings were obtained. This facilitates the fundamental understanding of deflocculation.

A decreasing ratio of bulk Ca^{2+} to K^+ is coupled with deflocculation and is proposed to be the cause for deflocculation under short-term low DO. To date, the mechanisms for deflocculation remain unclear. Results from this work suggest that the decrease in the ratio of extracellular Ca^{2+} to K^+ triggers deflocculation of sludge: The DO limitation causes an efflux of

cellular K^+ but an influx of extracellular Ca^{2+} across the cell membrane, thereby reducing the ratio of divalent to monovalent ions in the extracellular surroundings and giving rise to sludge deflocculation. Though specific cation fluxes have been proposed as a stress response of mammalian cells to oxygen shortage, no publications have been on examining the cation levels under the DO stress in an activated sludge system. Supported by the significant changes in extracellular K^+ and Ca^{2+} under the DO transient, this work is first to propose the involvement of cation fluxes in response to the DO limitation, leading to sludge deflocculation.

Deflocculation due to the transients of short-term low DO could be a physiological stress response at the metabolism level, which has not been specified previously. In comparison to the limited information on reversibility in published studies, this work reveals the fast reversible changes occurring within minutes to hours, including turbidity, SS, soluble proteins, removal of SCOD, levels of extracellular cations and particle size distribution. This suggests that responses of a mixed community to an short-term O_2 variation can be at a metabolic level, rather than a population shift to robust microbial species, for the latter one usually requires longer time (days) to take place.

Floc strength under the low DO is measured in this work, which has not been examined previously. This work shows increasing strength of remaining bioflocs in conjunction with deflocculation, which supports the model for bioflocs' structure, i.e., a weak outer layer with a strong inner layer.

This work demonstrates that deflocculation under the DO limitation is an erosion process of removing particles from a loose outer layer, rather than a process of breaking up bioflocs.

Findings from this work clarify that deflocculation mainly occurs on the outer layer of bioflocs, by which those particles loosely attached onto the surface are removed.

Externally adding Ca^{2+} manipulates the morphology of bioflocs by retaining the shell layer under the DO disturbances, which to some extent improves the treatment performance under the disturbance. The addition of extra Ca^{2+} prevents the carry-over of fine particles (< 1.5 μm) into treated effluents. Calcium enrichment is a relatively easy and inexpensive way to improve effluent quality under disturbances and should be taken into consideration for treatment plant operators.

CHAPTER 8 RECOMMENDATIONS FOR FUTURE WORK

Based on the present work, recommendations for future research needs are put forward, including:

i) Exploring a better technique for extracting cations from pellets of bioflocs

Protocols for extracting pellet cations need to be further developed, in order to better measure the cation levels within flocs and cells. In this work, the extraction of pellet cations was of low efficiency. As shown in the *E. coli* test, the measurable amount of pellet K^+ was far below the theoretical number. To completely lyse the cells and extract all cellular cations, optimizing the extraction technique is required, such as increasing the amounts of acids used, lengthening the extraction time, as well as using acidification followed by sonication. This aids in performing a better mass balance among pellet cations, bulk cations and total cations.

ii) Identifying the activity of specific ion channels using confocal laser scanning microscopy (CLSM)

Identifying or monitoring the activity of K^+ channels will provide a direct evidence of cation movements in responding to DO variations. Using CLSM to locate K^+ and Ca^{2+} channels on the cell membrane has been proved to be feasible in eukaryotic species (Bhatla et al., 2003; Panyi et al., 2003). Moyer and Stanton (2001) were able to track the changes in the expression of a specific chloride channel in mammalian cells under CLSM. It would be of interest using the similar technique to locate and monitor the activities of K^+ and Ca^{2+} channels in activated sludge under the DO variations. This will directly prove the involvement of ion transport systems in responding to the DO variations.

iii) Examining other possible mechanisms involved in the DO stress

Though unsuccessful attempt in demonstrating a clear profile of EPS proteins using SDS-PAGE, effort should be made on optimizing analytical conditions, such as staining reagents, sample loading volume, and pre-concentration of proteins. As discussed previously, another possible mechanism for deflocculation can be the release or activation of specific extracellular enzymes to degrade EPS proteins under the stress. Current results have not yet rejected this enzymatic mechanism. It is possible that cells may deploy multiple strategies for coping with the stress. Using gel electrophoresis to identify EPS protein has been reported by Higgins and Novak (1997). Future work can continue this direction, to examine the possible changes in protein profile under DO disturbances.

Examining the levels of Fe in mixed liquor sludge and wastewaters is necessary to clarify the involvement of reduction of Fe^{3+} in the deflocculation under short-term low DO. Though the reduction of Fe^{3+} have been proposed to be an insignificant factor in this study, identifying the amounts of Fe^{3+} and Fe^{2+} upon the DO disturbance would provide a direct evidence for ruling out the possibility of reduction of Fe^{3+} as a trigger for deflocculation.

iv) Clarifying the effects of selected chemicals on deterring deflocculation

Examining the effects of added $CaCl_2$, TEA chloride or glibenclamide independently will help to clarify their individual role in deterring deflocculation. In this work, addition of TEA chloride and glibenclamide were tested after introducing $CaCl_2$ into the system. However, before the addition of TEA chloride or glibenclamide, mixed liquor of activated sludge from the SBRs was not re-mixed and re-distributed. Thus, the effects of TEA chloride and glibenclamide on the activated sludge were not independent. It is unclear whether some

improvements (i.e., the formation of a denser inner layer, the decreasing number of bioflocs smaller than 12.5 μm) are due to the lately added chemical, or just a combining effect of all the chemicals in the system.

v) Studying the time scale of cation variations

If deflocculation under the short-term low DO is a protective strategy of microbial cells to cope with disturbances, and if the diffusion barriers from the EPS and outer-membrane of the cells cannot be eliminated in reality, it may be feasible to focus on how to predict or prevent the deflocculation in advance rather than how to amend the deflocculation after it happens.

Further investigation on time scales of the changes in bulk cations will provide an effective approach for preventing deflocculation. Current off-line sample analysis cannot quickly detect the changes in extracellular cations within a short time scale (e.g., seconds or minutes). A better technique is required for real-time monitoring the concentrations of extracellular cations. If the variations in extracellular cations are captured quickly, this may be able to serve as an alarm for the onset of deflocculation and prevent it in advance.

vi) Examining the redox potential and the threshold of DO concentrations for deflocculation

Redox potential is a good indicator for the overall oxidation/reduction state of the system. Except the DO level, the concentrations of other electron acceptors, such as nitrate and sulphate, were not measured in this study. The extent of deflocculation could be affected by the presence of these alternatives and their reduced compounds. Thus, testing the redox potential of

the system would be valuable in a better understanding of deflocculation under the short-term DO disturbance.

Rather than using pure N_2 as a purging gas, using a mixture of air and N_2 to reduce the DO levels in transient reactors will help to better locate the DO threshold for deflocculation. Exploring the threshold of DO concentrations is of operational importance in industry. In this work, the transients of short-term low DO were initiated by N_2 purging and the DO levels were reduced below 0.5 mg/L within 5 min. Thus, the threshold of the DO concentration causing the occurrence of deflocculation was masked by the sharp decrease. In the scenario of using a mixed purging gas, the rate of DO reduction should be slowed down, and the DO threshold can be located. In addition, the declining rate of DO levels in reality should be slower than that generated by N_2 purging. Therefore, using a mixture of air and N_2 will better simulate the DO disturbances in the real world.

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APPENDICES

Appendix A: Bottle Test Results

Approximately 400 mL of mixed liquor sample was collected from four operating sequence batch reactors (SBRs) treated with pulp and paper effluents in the lab. The samples were transferred into control and transient bottles. A control bottle was put into a shaker at 210 rpm and 23ºC. The transient bottle(s) was purged with N_2 in a low DO test, or was placed into an ice bath for a low temperature test, or added with NaCl or KCl in a cation test. Supernatant turbidity was measured along the experimental time. Results showed that a short-term low DO caused increasing turbidity of supernatants and was relatively easier to be manageable in the laboratory. Therefore, a short-term DO transient was chosen to be the disturbance investigated in this work;

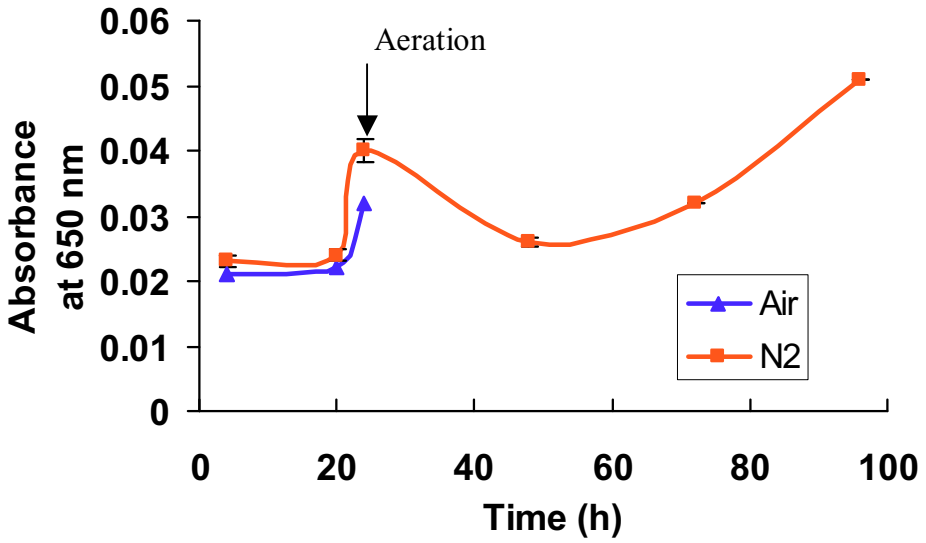


Figure A.1 Turbidity changes under DO variations: Only 5 min of N_2 purging was applied in the first 20 hours, there was insignificant difference between the control and transient samples; from the 20th to the 24th hours, 5 min of N_2 purging was applied every hour, transient samples showed a higher turbidity than the control sample. After re-aeration at the 24th hours, transient

turbidity declined for a period before going up again probably due to cell lyses. The error bars are +/- one standard deviation of the measurements.

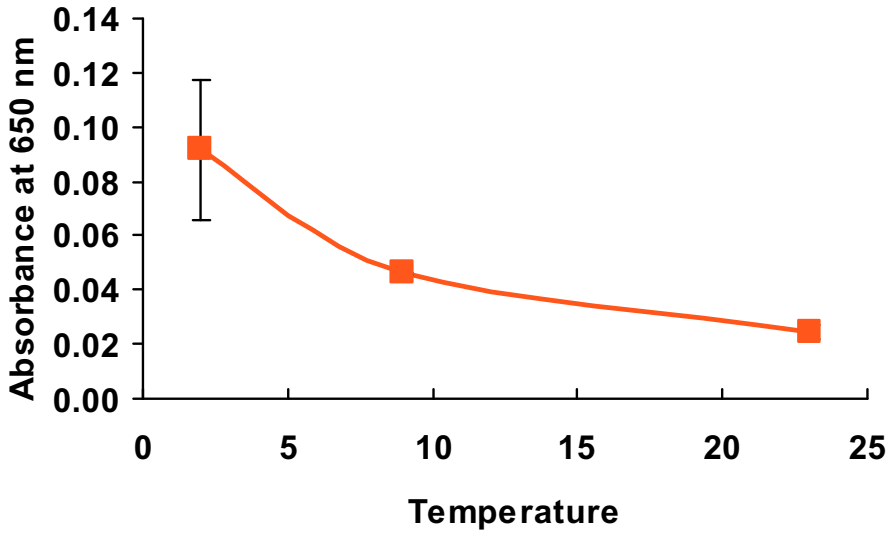


Figure A.2 Turbidity changes under temperature downshifts: Turbidity increased significantly when the temperature dropped below 4°C. The error bars are +/- one standard deviation of the measurements.

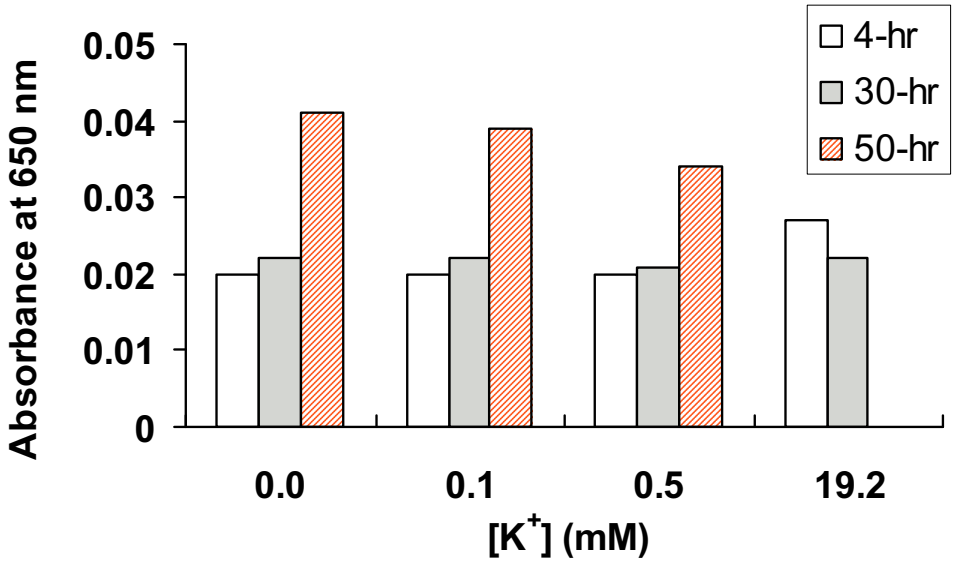


Figure A.3 Turbidity changes under the different concentrations of $[K^+]$: There was no clear correlation between turbidity changes upon the different levels of $[K^+]$. The increasing turbidity at t = 50 hours at different $[K^+]$ indicated cell lyses in both control and stressed samples.

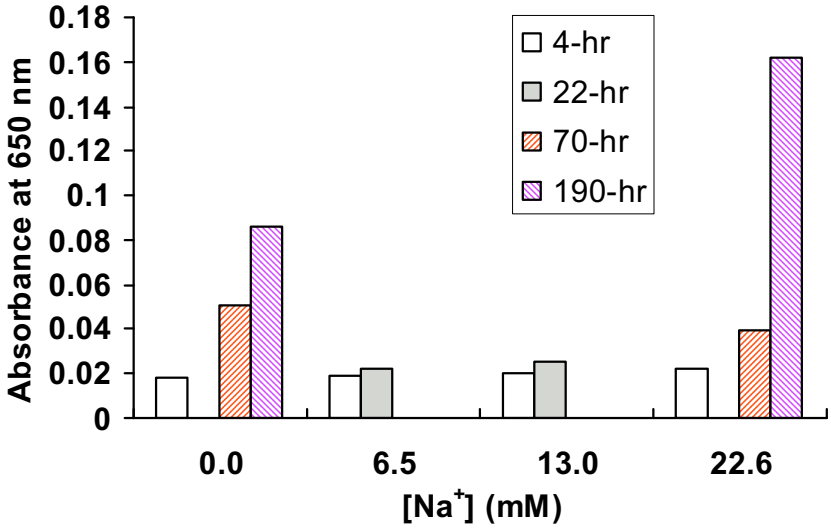


Figure A.4 Turbidity changes at the different levels of $[Na^+]$: There were no conclusive findings on the correlation between the concentrations of $[Na^+]$ and turbidity changes. Cell lyses might occur in both control and stressed samples after 70 hours, contributing to the turbidity increases.

Appendix B: Modified Lowry Method for Proteins and Humic Substances

The following procedures were modified based on Hartree (1972) and Frølund et al. (1995):

1. Prepare the following solutions:

    1) Solution A: Dissolve 0.2 g potassium sodium tartrate (Sigma-Aldrich, 99%) and 10 g Na_2CO_3 (Caledon Com., ≥99.5%) into 50 mL 1N NaOH (Caledon Com., ≥ 97%), and dilute the solution with nanopure water to 100 mL.

    2) Solution B: Dissolved 2 g potassium sodium tartrate and 1g $CuSO_4.5H_2O$ (Sigma-Aldrich, ≥ 98%) into 90 mL nanopure water, and add 10 mL 1N NaOH into the solution.

    3) Solution C: Dilute foli-Ciocalteu reagent (Sigma-Aldrich Com., 2N) with nanopure water at a ratio of 1: 15.

Solutions A and B can be stored at room temperature for up to 1 week. Solution C is made freshly every time right before the analysis.

2. Prepare standards for calibration curves:

    1) Proteins: Prepare a stock solution of Bovine Serum Albumin (BSA) at 1 mg/mL, and dilute to different concentrations from 25 mg/L to 250 mg/L using nanopure water.

    2) Humic substances: Prepare a stock solution of humic acid sodium salt at 1 mg/mL, and dilute to different levels from 25 mg/L to 250 mg/L using nanopure water.

3. Sample analysis:

    1) Take 2 x 1 mL unknown samples in two HACH tubes.

2) Add 0.9 mL solution A into each of them, vortex for 5 seconds (at medium intensity) and put the samples into a water bath at 50°C for 10 min.

3) Cool the samples to room temperature. Then, one of them is added with 0.1 mL solution B, vortex for 5 seconds and is left at room temperature for another 15 min.

4) Mildly vortex the samples (both added with and without solution B) while adding 3 mL solution C, continue vortexing the solution for another 10 seconds.

5) Put the samples into water batch at 50°C for 10 min.

6) Cool the samples till room temperature before taking absorbance readings at 650 nm.

7) The absorbance of the sample added with solution B is A_{total} whereas the absorbance of the sample without adding solution B is A_{blind}.

8) The absorbance for proteins is calculated as $A_{protein} = 1.25 (A_{total} - A_{blind})$; the absorbance for humic substances is calculated as $A_{humic} = A_{blind} - 0.2A_{protein}$.

9) For calibration curves of proteins and humic substances, repeat steps 1) to 6) using different concentrations of the standards for proteins and humic substances, draw the curves of absorbance readings vs concentrations.

Appendix C: Data over Acclimation Period

C-1: Mixed Liquor Suspended Solids

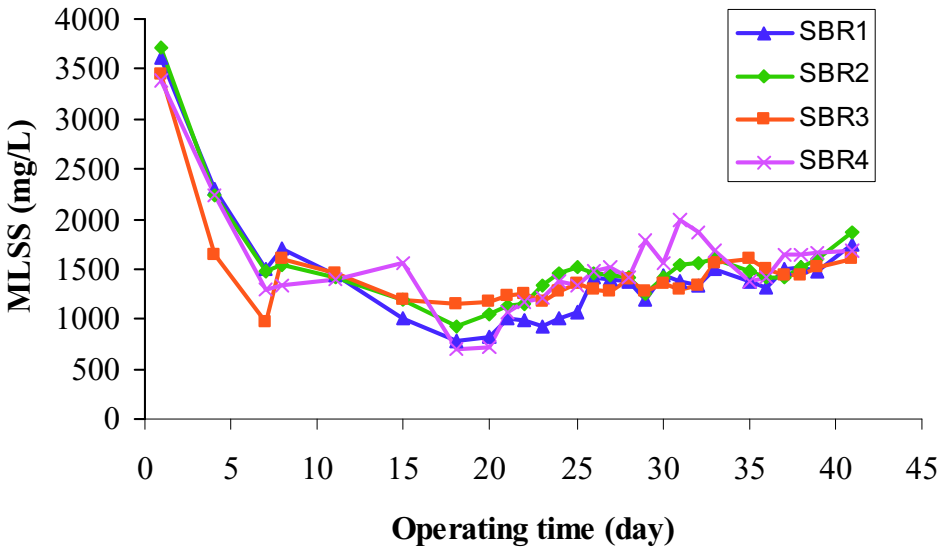


Figure C.1: Profile of MLSS over the acclimation period.

C-2: Removal of SCOD

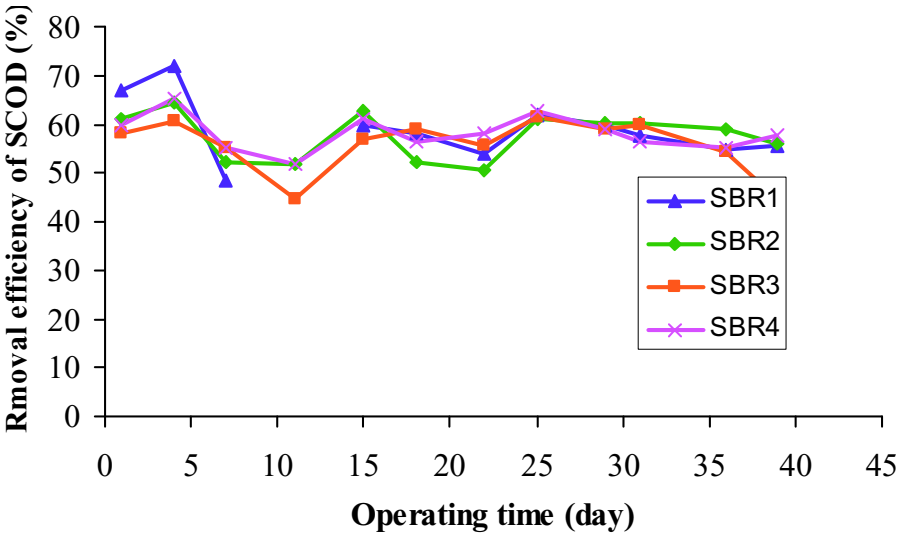


Figure C.2 Profile of SCOD over the acclimation period.

C-3: Effluent Suspended Solids

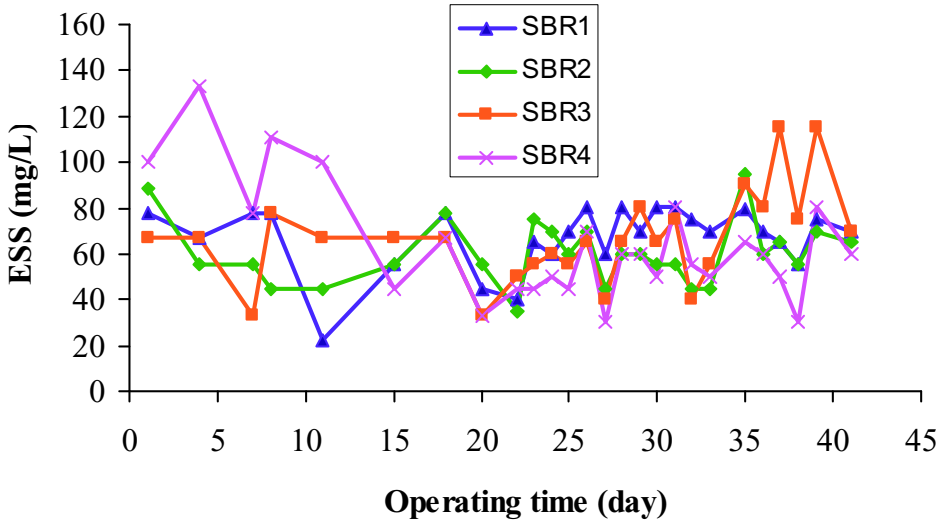


Figure C.3 Profile of ESS over the acclimation period.

C-4: Effluent Turbidity

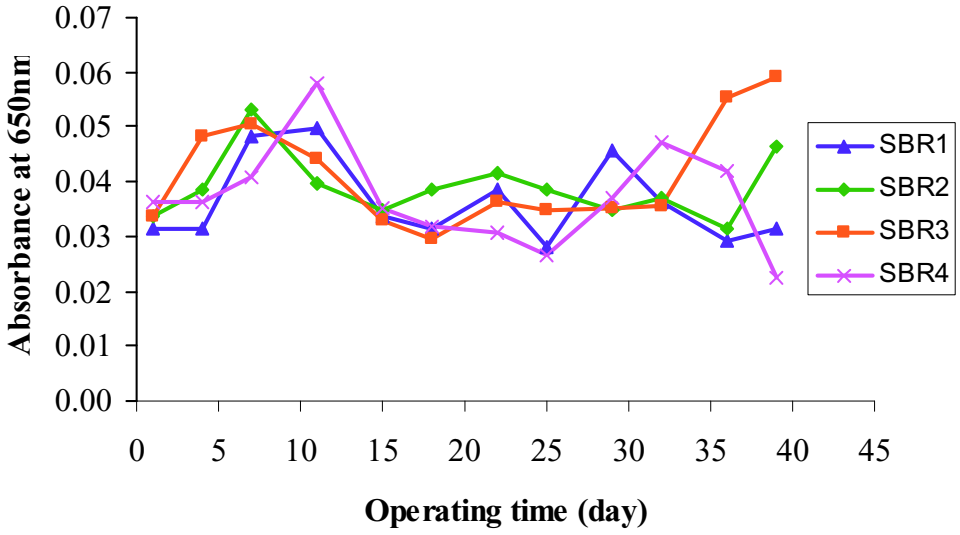


Figure C.4 Profile of effluent turbidity over the acclimation period.

Appendix D: Changes in Na^+ and Mg^{2+} upon the DO Variations

Overall, transients of short-term low DO have little impact on the changes in extracellular Na^+ and Mg^{2+}, as shown in the following results from batch and continuous experiments.

D-1: Batch Experimental Results

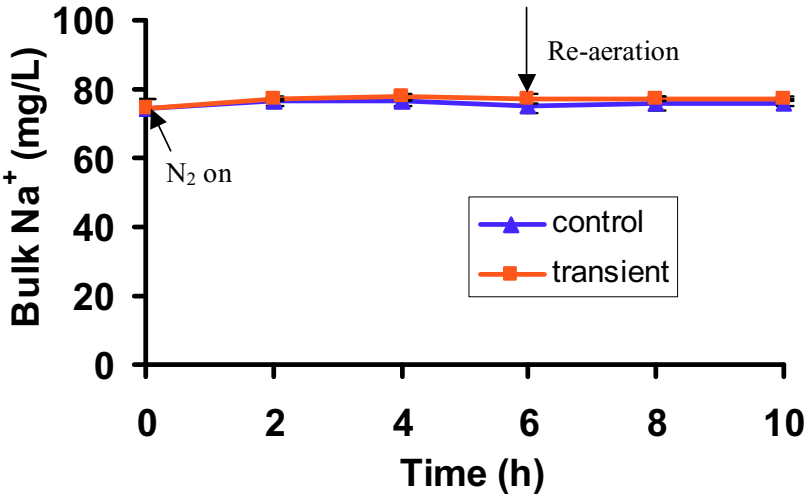


Figure D.1.1 Changes in bulk Na^+ in a typical DO test: There was insignificant difference between the control and transient data ($p = 0.12$).

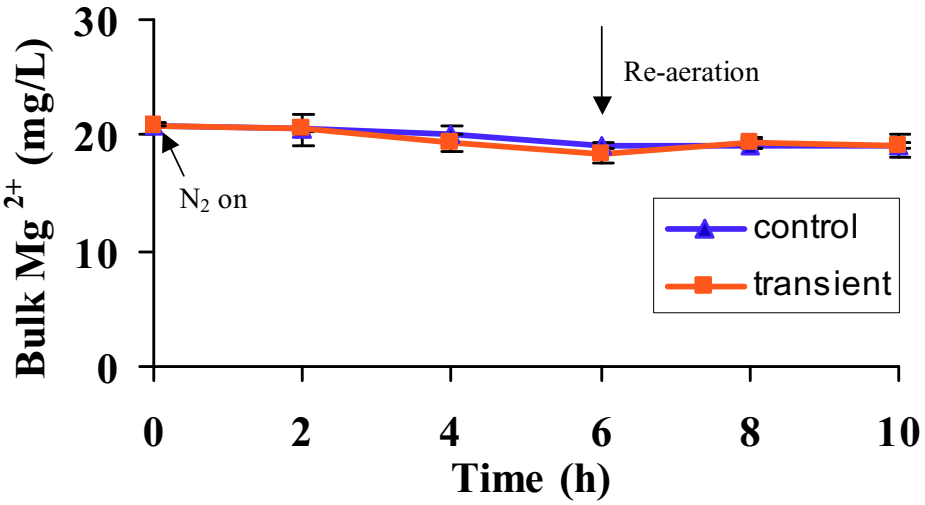


Figure D.1.2 Changes in bulk Mg^{2+} in a typical DO test: There was insignificant difference between the control and transient data (p = 0.20). The error bars are +/- one standard deviation of the measurements.

D-2: Continuous Experimental Results

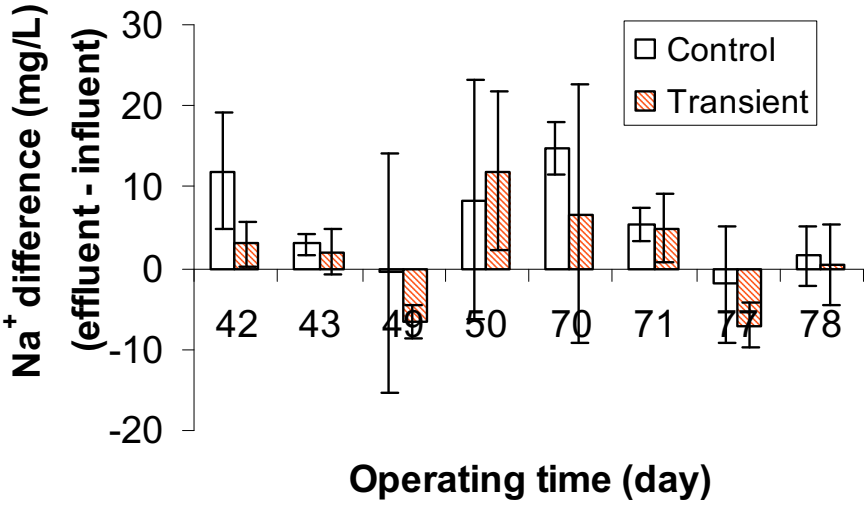


Figure D.2.1 Changes of Na^{+} difference upon DO variations: Transient days are 42, 49, 70 and 77. There was insignificant difference between the control and transient samples (p = 0.17). The error bars are +/- one standard deviation of the measurements.

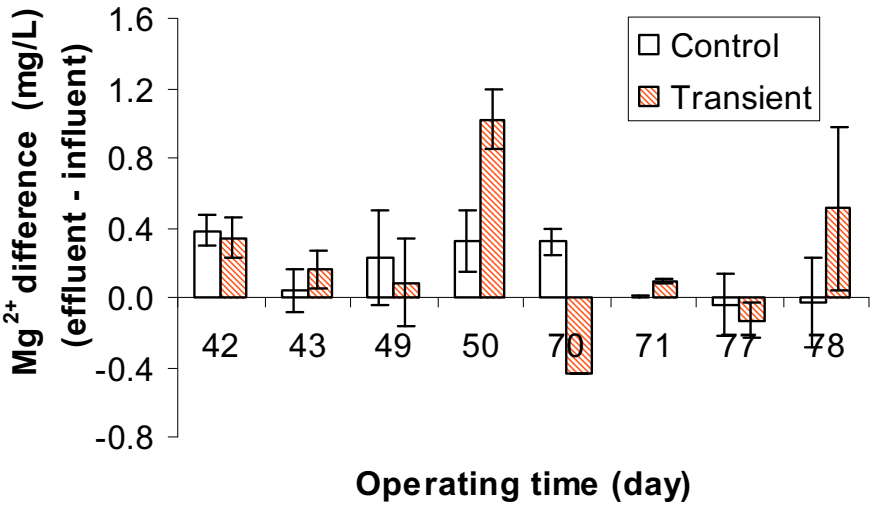


Figure D.2.2 Changes of Mg^{2+} difference upon DO variations: Transient days are 42, 49, 70 and 77. There was insignificant difference between the control and transient samples (p = 0.22). The error bars are +/- one standard deviation of the measurements.

D-3: Results from *E. coli* Tests

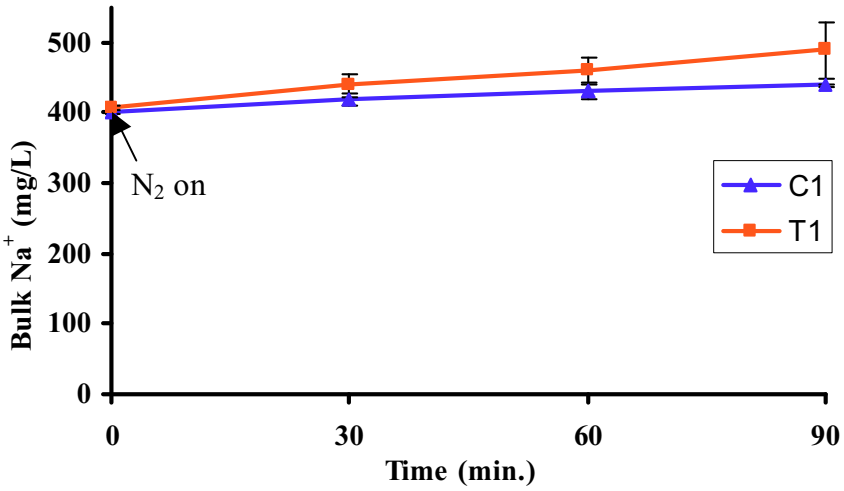


Figure D.3.1 Changes in bulk Na^{+} in a typical *E. coli* test: there was insignificant difference between the control and transient data (p = 0.09). The error bars are +/- one standard deviation of the measurements.

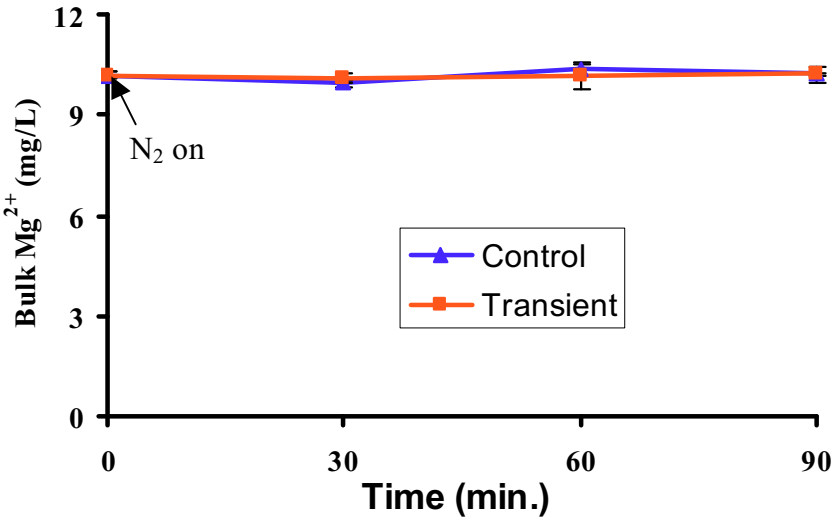


Figure D.3.2 Changes of bulk Mg^{2+} in a typical *E. coli* test: there was no significant difference between the control and transient data ($p = 0.24$). The error bars are +/- one standard deviation of the measurements.

Appendix E: Batch Results with pH Control

pH in one N_2 purging reactor was controlled using phosphate buffer solution. The pH profile of each reactor is depicted in Figures E.1. Changes in turbidity, SS and soluble proteins are presented in Figures E.2 to E.4. The difference between reactors "Air" and "Air + pH control" represents the effect of buffer solution on activated sludge. A comparison between reactors "N_2" and "N_2 + pH control" demonstrates the effect of pH changes on the sludge (caused by the reduction of produced CO_2 and continuous CO_2 stripping-off). As well, reactor "Air + pH control" serves as a reference to reactor "N_2 + pH control", to distinguish the pure low DO effect.

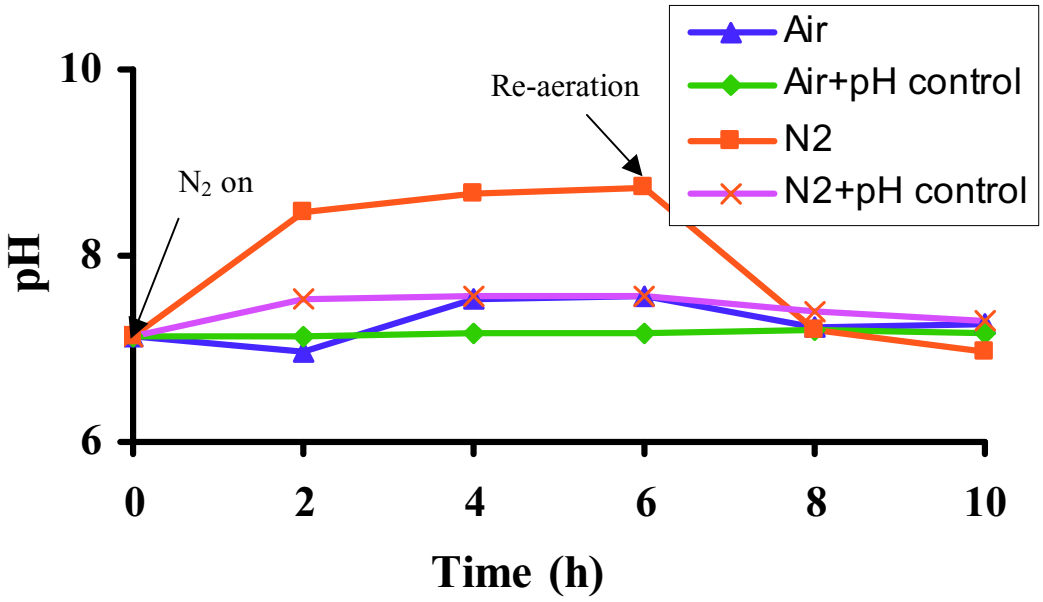


Figure E.1 pH profile in a typical DO test with pH control.

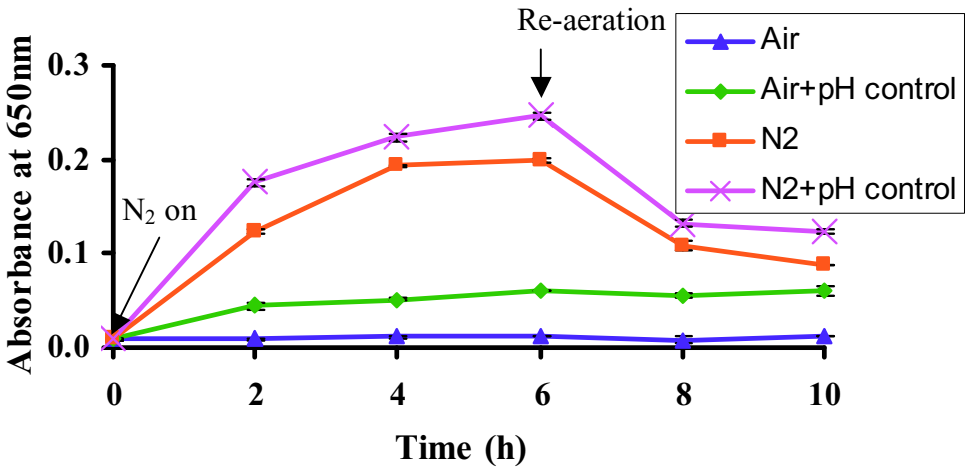


Figure E.2 Turbidity changes under the DO stress with pH control: Both DO-stressed reactor had the higher turbidity than the control ones. Reactor "N_2 + pH control" has the highest turbidity.

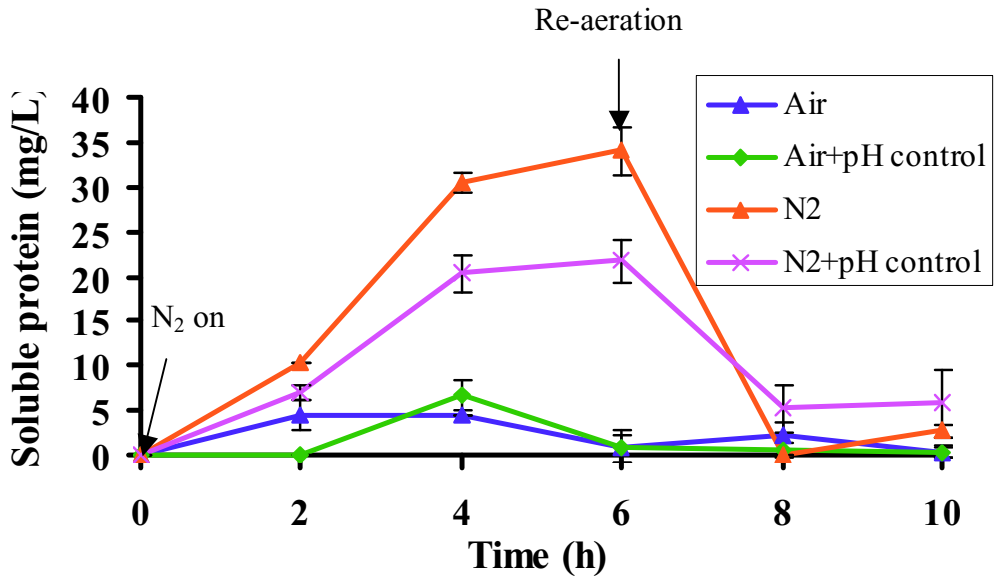
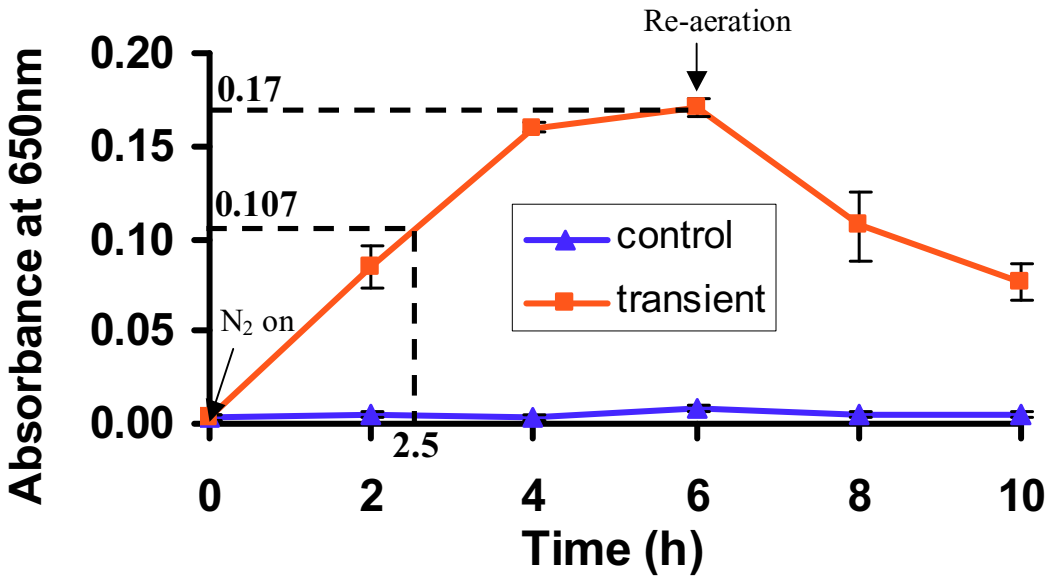


Figure E.3 Changes in soluble proteins in a typical DO test with pH control. The error bars are +/- one standard deviation of the measurements.

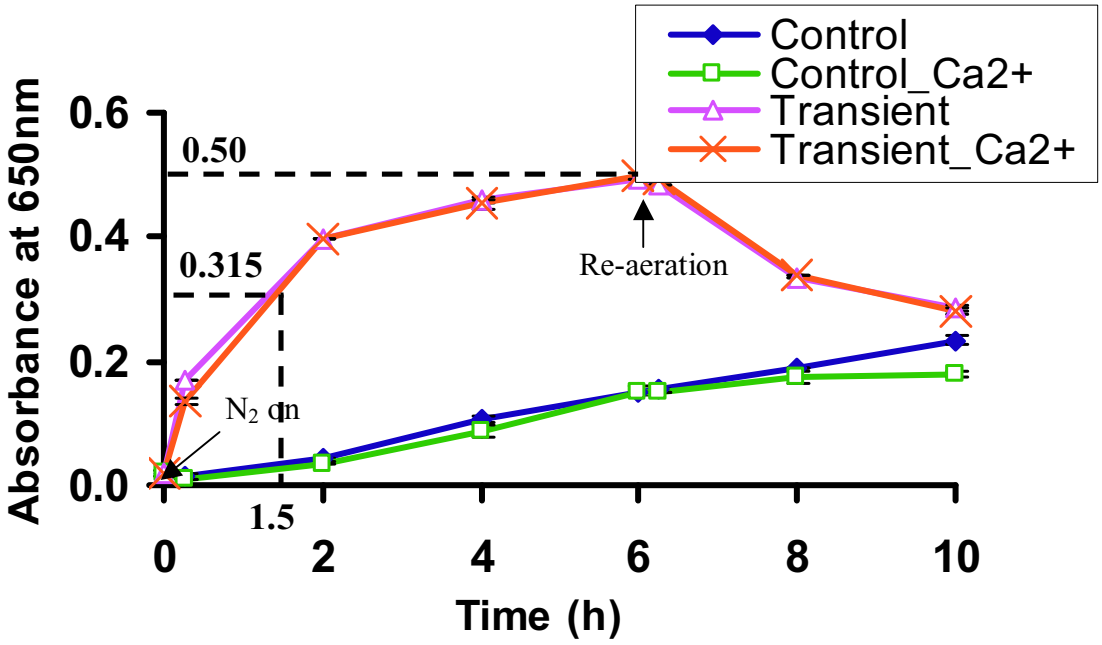
Appendix F: Sample Calculations of Time Constants

It is assumed that the profile of transient turbidity upon the DO limitation (t=0 to 6 hours) follows a first-order response. Time constant refers to the time when the turbidity reaches 63% of its final value.

An example of calculation based on Figures 4.1 and 4.16 is illustrated in Figure F.1. For the turbidity profile in Figure (a), the final value of turbidity is approximately 0.17, so the time to reach the value of 0.107 (0.63 x 0.17) is around 2.5 hours. For the turbidity profile in Figure (b), the final value of turbidity is approximately 0.5, thus the time to reach the value of 0.315 (0.63 x 0.5) is around 1.5 hours.



(a)



(b)

Figure F.1 Sample calculations of time constants of turbidity from different experiments.

Appendix G: Comparison Between Temperature and DO Disturbances

The effects of temperature upshifts from 30 to 45°C on the changes in extracellular K^+, Na^+, Ca^{2+} and Mg^{2+} were examined as an undergraduate thesis (Chmiel, 2006), as well as to compared the cation changes to the ones in DO batch experiments on activated sludge. Following figures are the main observations from the comparison. Time zero in the temperature tests was the onset of a shift of 15°C in triplicate reactors in comparison to a control reactor (maintained at 30°C). Time zero in the DO tests was the onset of N_2 purging in the transient reactors. In short, compared to the short-term low DO, the temperature transient had a slower effect on the changes in turbidity, bulk K^+ and Ca^{2+}. The degree of turbidity changes appeared to be correlated with the degree of changes in bulk cations. The following Figures are of courtesy of Monika Chmiel.

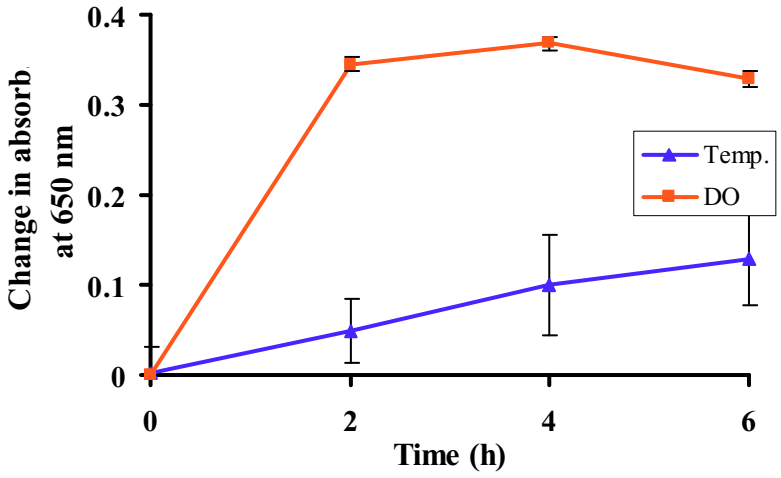


Figure G.1 Comparison of turbidity changes: DO transients caused much higher turbidity in the first 2 hours, probably due to the sharp decline in the DO levels.

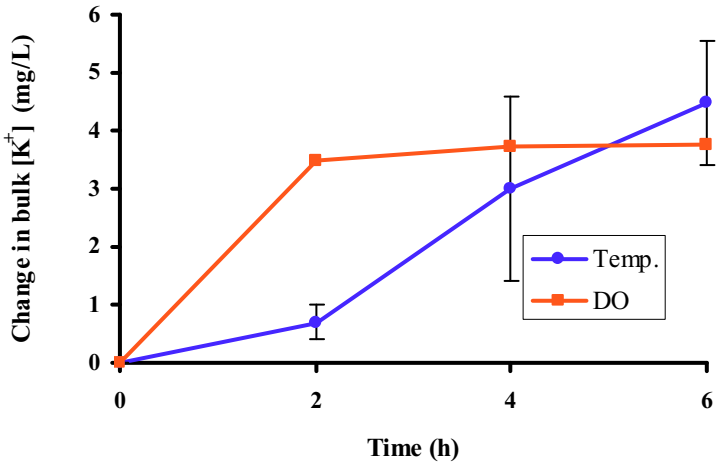


Figure G.2 Comparison of changes in bulk K^+: In the first 2 hours, the increase in bulk K^+ under DO limitation was much higher than that under temperature upshift.

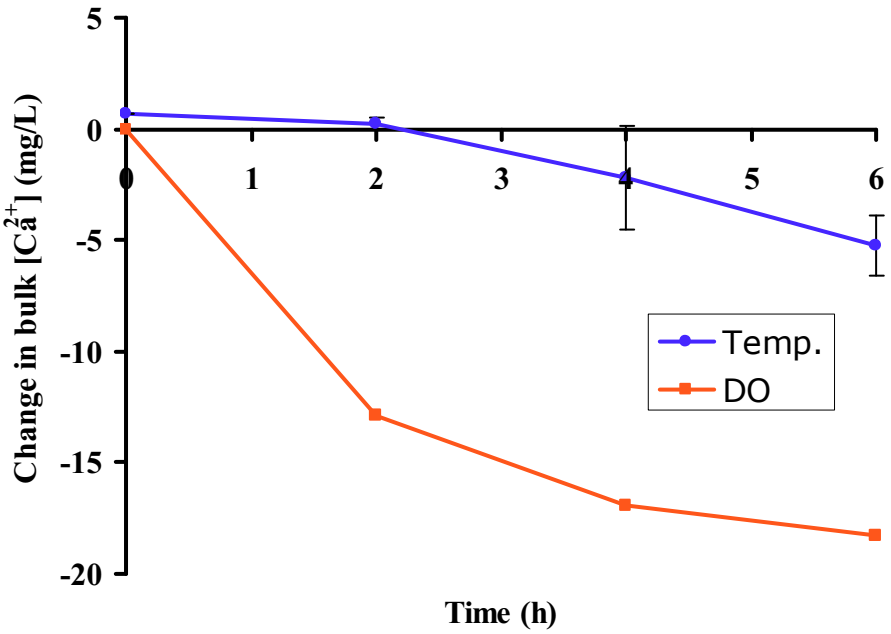


Figure G.3 Comparison of the changes in bulk Ca^{2+}: Changes in bulk Ca^{2+} under temperature upshift were lower than those under DO limitation over the experimental time.