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**UNDERSTANDING THE STABILITY OF CYCLIC
AEROBIC-ANOXIC CONDITIONED SLUDGE FLOCS
UNDER DEFLOCCULATIVE STRESSES**

AKSHAYKUMAR SURESH

**INTERDISCIPLINARY GRADUATE SCHOOL
NANYANG ENVIRONMENT & WATER RESEARCH INSTITUTE**

2019

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AEROBIC-ANOXIC CONDITIONED SLUDGE FLOCS
UNDER DEFLOCCULATIVE STRESSES**

AKSHAYKUMAR SURESH

Interdisciplinary Graduate School

Nanyang Environment & Water Research Institute (NEWRI)

A thesis submitted to the Nanyang Technological University

in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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.....S. Akshay K.....

AKSHAYKUMAR SURESH

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I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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PROEN NG WUN JERN

AUTHORSHIP ATTRIBUTION STATEMENT

*(B) This thesis contains material from 3 papers published in the following peer-reviewed journal(s) / from papers accepted at conferences in which I am listed as an author.

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The contributions of the co-authors are as follows:

- Prof Ng Wun Jern and Dr. Xin Gao (representing Grundfos Holdings A/S) provided the initial plan for review topics, revised and edited the overall manuscript.
- I prepared several sections including "Floc bio-components", "DO", "Temperature", "Feeding Regimes", "F/M ratio" while also contributing to other sections of the reviews. I also performed the overall compilation of the topics into a review manuscript with the aid of Dr. Ewa Grygolowicz-Pawlak.
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- Dr. Santosh Pathak contributed to the "SRT" and "F/M ratio" sub-sections and aided in the revision of the manuscript.
- Dr. Leong Soon Poh contributed to the "Indicators of Flocculation and Deflocculation" sub-section and aided in the revision of the manuscript.
- Dr. Maszenan bin Abdul Majid contributed to the manuscript revisions and edits.
- Dr. Dominik Dominiak and Dr. Thomas Vistisen Bugge (representing Grundfos Holdings A/S) contributed to the "Indicators of Flocculation and Deflocculation" sub-sections and aided in the editing and revision of the manuscript.

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- I performed all the experiments in tandem with Dr. Ewa Grygolwicz-Pawlak. I also prepared the manuscript drafts.
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- Dr. Leong Soon Poh helped with collection of sludge samples from the treatment plants and also aided in the revision of manuscripts.
- Dr. Maszenan bin Abdul Majid contributed to the manuscript revisions and edits.
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- Prof Ng Wun Jern was involved in planning of the experimental work and edited the manuscript drafts.
- I performed all the experiments and also prepared the manuscript drafts.
- Dr. Chaozhi Pan aided in the revision and editing of manuscript drafts.

20/Jan/2020

Date

S. Akshay

AKSHAYKUMAR SURESH

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	i
ABSTRACT.....	viii
TABLE OF FIGURES.....	x
LIST OF TABLES	xiv
ABBREVIATIONS	xv
CHAPTER 1	1
1.1 Introduction	1
1.2 Aims and Objectives.....	3
1.3 Thesis Outline	7
CHAPTER 2	9
Literature Review	9
2.1 Floc bio-composition and its role in the biological flocculation process	9
2.2 Physico-chemical influence on the bio-components and their subsequent effects on bio-flocculation	12
2.2.1 Temperature.....	12
2.2.2 Ionic composition.....	14
2.2.3 Toxins.....	18
2.3 Influence of process parameters on the sludge flocs and biological flocculation.....	21
2.3.1 Dissolved oxygen	21
2.3.2 Feeding regimes	24
2.3.3 Solids retention time.....	25
2.3.4 Right nutrients balance	27
2.4 Indicators of flocculation and deflocculation.....	30
2.5 Summary and Research Gaps	33

CHAPTER 3	37
Analytical Methods	37
3.1 Total suspended solids (TSS) and volatile suspended solids (VSS) measurements	37
3.2 Dissolved oxygen carbon (DOC) and soluble chemical oxygen demand (COD) measurements.....	37
3.3 DO and ionic measurements.....	37
3.4 Turbidity measurements.....	38
3.5 Floc size measurements.....	38
3.6 SMP & bound EPS extraction protocol	38
3.7 Nitrate and nitrite measurements	39
3.8 Colorimetric measurements	39
3.9 FEEM and synchronous fluorescence analysis.....	39
CHAPTER 4	43
Evidence for the resistance of activated sludge flocs to deflocculative DO stress.....	43
4.1 Introduction	43
4.2 Materials and methods.....	45
4.2.1 Sludge samples	45
4.2.2 Experimental protocol.....	47
4.2.3 Analytical Methods.....	48
4.3 Results	49
4.3.1 SBR sludge.....	49
4.3.2 WWTP 1 sludge	54
4.3.3 WWTP 2 sludge	58
4.4 Discussions	62
4.5 Conclusions	67
CHAPTER 5	68

Sodium azide inhibition of microbial activities and impact on sludge floc destabilization.....	68
5.1 Introduction	68
5.2 Materials and methods.....	70
5.2.1 Sludge samples	70
5.2.2 Experimental setups	70
5.2.3 Analytical Methods.....	73
5.3 Results	73
5.3.1 Influence of sodium azide on flocculation under high DO conditions ...	73
5.3.2 Interactions between sodium azide and bound EPS.....	81
5.3.3 Sodium azide induced deflocculation under low DO conditions	83
5.4 Discussions	89
5.5 Conclusions	92
CHAPTER 6	93
Impact of cation chelation on destabilization of the sludge floc ecosystem	93
6.1 Introduction	93
6.2 Materials and Methods	95
6.2.1 Sludge samples	95
6.2.2 Experimental protocols	95
6.2.3 Analytical Methods.....	97
6.3 Results	97
6.3.1 Influence of EDTA on floc stability and integrity	97
6.3.2 Influence of calcium complexed EDTA on the sludge flocs	106
6.4 Discussions	114
6.5 Conclusions	116
CHAPTER 7	118
Overall Conclusions and Practical Implications	118

7.1 Absence of deflocculation under low DO conditions and its practical implications	118
7.2 Importance of long-term conditioning on the stability of sludge floc ecosystem.....	122
REFERENCES.....	124

ABSTRACT

Biological wastewater treatment with activated sludge followed by a liquid-solids separation step has been an established process for over a century. Effective liquid-solids separation can be made through biological or chemical flocculation. Biological flocculation involves the aggregation of organic and inorganic components into flocs through the action of microbes and their by-products, i.e. extracellular polymeric substances (EPS). Adverse conditions can promote floc break-up, i.e. deflocculation, reducing effluent quality and increasing overall treatment costs. It is essential to have a better understanding of the biological flocculation process and factors influencing it, especially in cyclically conditioned (aerobic/anoxic/anaerobic) treatment processes.

The exposure to low dissolved oxygen (DO) (<0.5 mg/L) conditions has been reported to promote deflocculation. However, in this dissertation studies, sludge from three different sources repeatedly showed a lack of deflocculation even after 24 hours subject to low DO and substrate depletion. Divalent cationic interactions, high bound EPS content and the activities of facultative bacterial communities were factors argued to have contributed to increased floc integrity, and which helped mitigate the deflocculation under low DO conditions. The importance of facultative bacterial communities on floc maintenance and mitigation of deflocculation under low DO conditions was confirmed through microbial inhibition experiments. Deflocculation, in the form of floc erosion, was observed under low DO conditions only when microbial inhibitory conditions were promoted using a mitochondrial uncoupler, sodium azide. Sodium azide also exhibited interactive tendencies with the EPS, which was confirmed through EPS fluorescence quenching studies and this was another mechanism through which floc integrity could be influenced. It was the microbial communities, their activities and metabolic by-products (EPS), and not necessarily the short-term DO

transients, that influenced the flocculation state. The role of cations and their interactions in the stabilization of floc ecosystem was also confirmed when deflocculation occurred upon interaction of sludge samples with a chelating agent, ethylenediaminetetraacetic acid (EDTA). The chelation of calcium and magnesium ions from the floc body not only reduced the floc stabilizing ionic interactions, but also resulted in hampered microbial viability and activities. Consequently, irreversible deflocculation was observed above a threshold concentration of the chelating agent. Pre-complexation of EDTA with calcium ions counteracted its cation chelating, anti-bacterial and deflocculative tendencies. This highlighted the cations played a more important role in the overall floc ecosystem than previously considered, which helped maintain SBR sludge floc stability and integrity under different DO conditions.

The results of this study confirmed the importance of overall microbial activities (including facultative bacteria) and cationic interactions in the maintenance of the floc ecosystem while disagreeing with the importance of short-term low DO transients. Unless a considerable adversarial stress was applied on the microbial communities, the EPS or cationic interactions, the sludge flocs would remain stable in a short-time disruption event. Thus, biological floc maintenance is a complex process that should be investigated relative to the conditions the flocs are most exposed to on a long-term basis.

TABLE OF FIGURES

Figure 2.1: A schematic of the floc structure held together by the bound EPS and bacteria.....	11
Figure 2.2: Major phenomena governing EPS interactions: a) dissociation of functional groups (adapted from [67], b) repulsive electrostatic vs. attractive Van der Waal forces and c) multivalent cation bridging theory (adapted from [68, 69]).	15
Figure 3.1: The various fluorophore regions in the FEEM contour graph (adapted from [186]).....	41
Figure 4.1: Changes in (a) DO, (b) ammonium, (c) pH and (d) turbidity of SBR sludge samples (AN, AN-OX, OX and H-OX) during the batch test.	50
Figure 4.2: Measurements of the SBR sludge samples at the end of the batch test: (a) particle-size distribution; (b) chemical composition of SMP, LB EPS, and TB EPS fractions according to colorimetry; and (c) chemical composition of SMP, LB EPS, and TB EPS fractions according to FEEM measurements. The FEEM measurements were performed at 10 nm excitation/emission slits for SMP and LB EPS fractions. For the TB EPS fractions, FEEM measurements were performed at 5 nm excitation/emission slits.	53
Figure 4.3: Changes in (a) DO, (b) ammonium, (c) pH and (d) turbidity of WWTP 1 sludge samples (AN, AN-OX, OX and H-OX) during the batch test.	55
Figure 4.4: Measurements of the WWTP 1 sludge samples at the end of the batch test: (a) particle-size distribution; (b) chemical composition of SMP, LB EPS, and TB EPS fractions according to colorimetry; and (c) chemical composition of SMP, LB EPS, and TB EPS fractions according to FEEM measurements. The FEEM measurements were performed at 10 nm excitation/emission slits for SMP and LB EPS fractions. For	

the TB EPS fractions, FEEM measurements were performed at 5 nm excitation/emission slits.	57
Figure 4.5: Changes in (a) DO, (b) ammonium, (c) pH and (d) turbidity of WWTP 2 sludge samples (AN, AN-OX, OX and H-OX) during the batch test.	60
Figure 4.6: Measurements of the WWTP 2 sludge samples at the end of the batch test: (a) particle-size distribution; (b) chemical composition of SMP, LB EPS, and TB EPS fractions according to colorimetry; and (c) chemical composition of SMP, LB EPS, and TB EPS fractions according to FEEM measurements. The FEEM measurements were performed at 10 nm excitation/emission slits for SMP and LB EPS fractions. For the TB EPS fractions, FEEM measurements were performed at 5 nm excitation/emission slits.	61
Figure 5.1: Changes in (a) ammonium, (b) dissolved organic carbon, (c) pH, (d) DO, (e) supernatant turbidity, (f) potassium, (g) nitrate and (h) nitrite contents of SBR sludge samples subjected to various azide concentrations.	76
Figure 5.3: Measurement of the SMP and bound EPS content by (a) colorimetry, (b) FEEM analysis at the end of the batch test. For the FEEM and synchronous fluorescence analysis, SMP, LB and TB EPS contents were measured at different voltages making only intra-fraction analysis feasible (PN-Protein, HA-Humic acid, AA-Amino acid, PS-Polysaccharides and FA-Fulvic acid).	79
Figure 5.4: The total fluorescent EPS intensities (a, b) and UV-absorption spectra (c, d) of TB EPS (a and c) and LB EPS (b and d) fractions interacted with different azide concentrations.	82

Figure 5.5: Changes in the (a) supernatant turbidity, (b) potassium, (c) ammonium and (d) DO content of SBR sludge samples subjected to different batch test conditions (OX, AN and AZ-AN).	85
Figure 5.6: Measurement of (a) mean floc sizes, and (b) floc size distribution measured at the end of the batch test.....	86
Figure 5.7: Measurement of the SMP and bound EPS content by (a) colorimetry and (b) FEEM analysis at the end of the batch test. For the FEEM analysis, SMP, LB and TB EPS contents were measured at different voltages making only intra-fraction analysis feasible (PN-Protein, HA-Humic acid, AA-Amino acid, PS-Polysaccharides and FA-Fulvic acid).	88
Figure 6.1: Profiles of (a) supernatant turbidity changes, (b) mean floc size and (c) floc size distribution of samples subjected to different EDTA concentrations. The mean floc size and floc size distribution measurements were performed at the end of the batch test.	99
Figure 6.2: Measurement of chemical composition of EPS by (a) colorimetry, (b) FEEM analysis and (c, d and e) synchronous fluorescence analysis (with a $\Delta\lambda = 60$ nm) in the various EPS fractions.	102
Figure 6.3: Profiles of (a) ammonium, (b) soluble COD, (c) nitrate, (d) potassium, (e) calcium, (e) magnesium and (e) iron content of samples subjected to various EDTA concentrations. The cationic content in the different EPS fractions were assessed at the end of the batch test.	105
Figure 6.4: Profiles of (a) supernatant turbidity changes, (b) mean floc size and (c) floc size distribution of samples subjected to non-complexed and Ca-complexed EDTA	

additions. The mean floc size and floc size distribution measurements were performed at the end of the batch test.....	107
Figure 6.5: Measurement of chemical composition of EPS by (a) colorimetry, (b) FEEM analysis and (c, d and e) synchronous fluorescence analysis (with a $\Delta\lambda = 60$ nm) in the various EPS fractions.	110
Figure 6.6: Profiles of (a) ammonium, (b) soluble COD, (c) nitrate, (d) potassium, (e) calcium, and magnesium content of non-complexed EDTA and Ca-EDTA spiked samples. The cationic content in the bound EPS fractions were assessed at the end of the batch test.	113

LIST OF TABLES

Table 2.1: Interconnected factors influencing biological sludge flocculation.....	29
Table 3.1: The FEEM regions corresponding to the fluorescing EPS components.....	40

ABBREVIATIONS

EPS	Extracellular polymeric substances
SMP	Soluble Microbial Products
LB EPS	Loosely bound EPS
TB EPS	Tightly bound EPS
DNase	Deoxyribonuclease
eDNA	Extracellular DNA
DO	Dissolved oxygen
SRT	Solids retention time
ORP	Oxidation-reduction potential
SVI	Sludge volume index
TSS	Total Suspended Solids
VSS	Volatile Suspended Solids
COD	Chemical Oxygen Demand
BOD	Biological Oxygen Demand
DOC	Dissolved Organic Carbon
MLSS	Mixed Liquor Suspension Solids
F/M	Food to microorganisms ratio
WWTP	Wastewater Treatment Plant

CHAPTER 1

CHAPTER 1

1.1 Introduction

The activated sludge process has been used for over a century and remains one of the most widely used biological wastewater treatment processes since its introduction by Arden and Lockett [1, 2]. Many physical, chemical and biological properties of the activated sludge had been studied in attempts to better understand functionality and hence facilitate process control.

The conventional activated sludge (CAS) process is typically deployed for the conversion of pollutants such as organic carbon, nitrogen and phosphorus compounds [3-5]. Organic pollutants are broken down and utilized by the microorganisms under aerobic conditions for their growth and metabolism. Nitrogen removal is typically achieved in a two steps process: aerobic conversion of ammonium to nitrate (nitrification) followed by nitrate reduction to nitrogen gas under anoxic conditions (denitrification). This has necessitated the need for cyclical activated sludge system where sequential aerobic/anoxic processes are employed to achieve effective nitrogen removal [6]. After these biological processes, sludge is settled and separated from the supernatant in the clarifier to produce a clarified effluent for discharge or post treatment. A fraction of the settled sludge is recycled for treatment of the incoming flow of influent. Clarified effluent without excessive solids carry over and sludge solids with short settling time are two important and desirable characteristics of the activated sludge process. Both of these characteristics are dependent on effective flocculation, which includes the formation of flocs that are larger, stronger, denser and more hydrophobic [7-9]. Biological flocculation is an ability exhibited by certain

CHAPTER 1

microorganisms (mainly bacteria) to form floccular aggregates. The bacterial communities engage in floc formation to protect themselves from predators and other environmental stresses [10]. This biological flocculation is partially induced by the synthesis of extracellular polymeric substances (EPS), which promote the necessary interactions required to hold the flocs together. The synthesis of proteins by the microbes are known to increase floc hydrophobicity and hydrophobic interactions. The flocculating tendencies of certain groups of bacterial communities are exploited in the biological treatment processes to enable both removal of organics and separation of clarified effluent from the remaining solids. Therefore, biological wastewater treatment processes are geared towards the cultivation and proliferation of floc-forming bacteria to allow for the most efficient biological flocculation. However, both conventional and cyclic activated sludge processes are susceptible to fluctuations in chemical and physical conditions, which may adversely impact biological flocculation capabilities. Reports in literature have stated short-term transient conditions (less than 4 hours) can adversely influence the floc structures and bring about deflocculation [11, 12]. Deflocculation causes effluent quality deterioration and this would then impose heavier demands on post-treatment processes, and thereby escalating the overall cost of treatment. Field experience had indicated that the mitigation of deflocculation is always more energy and chemicals intensive than maintaining good flocculation.

A common practice to enhance flocculation and sludge settling in the WWTP is to dose chemical coagulants. Coagulation is fast acting and improvements in effluent quality can be quickly observed. However, there are limitations to coagulation; water pH and hardness are two parameters that affect the efficiency of chemical coagulation. Furthermore, the coagulant is an additional cost and un-reacted coagulant is often

CHAPTER 1

undesirable in tertiary treatment operations (e.g. you do not want carried-over iron in RO [13]). Furthermore, when external flocculation aids are regularly used, the selective pressure for flocculating microorganisms will be lost creating more long-term ramifications. As flocculating microorganisms are progressively lost, the need for flocculation aids will increase. This would not only increase treatment cost but also increase the amount of waste sludge produced, and its disposal would increase overall costs.

Unfortunately, biological flocculation is a complex process, which is influenced by many factors and is typically more difficult to control than chemical coagulation and flocculation. With the prevalence of more cyclic processes in the treatment of different types of wastewaters, it is essential to reconsider and reevaluate the knowledge regarding the biological flocculation process and the factors influencing it. This will allow for more up to date information on the biological flocculation process, the understanding of which will allow researchers and WWTP engineers to develop better strategies for maintaining strong flocs and reducing the propensity of the sludge to deflocculate.

1.2 Aims and Objectives

Among the important factors influencing the biological flocculation process, the dissolved oxygen (DO) concentration is especially important considering their impact on the type of microbial growth and their metabolic activities [11, 12, 14, 15]. Aerobic, cyclic anoxic-aerobic and anaerobic conditions will bring about the prevalence of specific type of bacteria capable of respiring under these conditions [16]. With regards to the biological flocculation, important groups of bacteria, including the

CHAPTER 1

floc formers and filamentous bacteria, have specific DO requirements for their growth, propagation and continued viability. Considering the importance of microbial communities, many studies have been conducted to assess the influence of DO on the biological flocculation process. These studies have reported DO transients, even short-term (less than 1-4 hours), have a significant effect on floc integrity and stability. Deflocculation occurred in sludges subjected to short-term low DO (<0.5 mg/L) or anaerobic conditions [14, 15]. This was speculated to be due to the influence of DO transients on the microbial communities and their by-products, i.e. extracellular polymeric substances. The low DO conditions in these studies were brought about by purging the sludge samples with consistent supply of nitrogen gas. They reported the occurrence of deflocculation as early as 2 hours of the DO conditioning, in terms of increased supernatant turbidity and soluble microbial products (SMPs).

However, the same deflocculative symptoms were not observed in the preliminary experimental works conducted in our lab with SBR sludge samples. When similar experimental methods were utilized to bring about aerobic and low DO (<0.5 mg/L) conditions, i.e. with gas purging, low DO deflocculation, in terms of supernatant turbidity, was not observed even after 6 hours. Significant turbidity increase was observed in the nitrogen purged samples only after overnight purging of nitrogen (more than 18 hours). It has to be noted here the purging of nitrogen gas to bring about low DO conditions can bring about additional shear stress. This can exacerbate the adverse impact on floc integrity and stability under the low DO conditions. The deflocculation was observed only after overnight purging, which could have also been due to other factors like substrate and nutrient depletion. When subsequent studies were performed to establish repeatability, higher standard deviations were observed in

CHAPTER 1

the supernatant turbidity values of low DO conditioned duplicates. This was expected to be a side effect of the additional shear stress brought about by purging. These preliminary results indicated the low DO deflocculation might not be a universal phenomenon and the effect of experimental and analytical methods on floc integrity should not be discounted. Thus, first objective of this dissertation study was to more accurately evaluate the effect of the DO concentration on sludge flocs while minimizing the other variables that can affect the outcome like shear stress (due to bubbling) and substrate depletion. Batch tests were conducted with sludges from three different sources (1 laboratory cultivated SBR sludge and 2 WWTP sludges) to assess the reported universality of the phenomenon of low DO influenced deflocculation.

After evaluating the effects of the DO levels on the sludge flocs and flocculation, the second phase of the dissertation study involved the assessment of the importance of microbial viability and activity in the maintenance of floc integrity and stability. There are reports in literature stating the importance of microbial communities and their activities, especially that of aerobes, in floc maintenance and mitigation of deflocculation [11, 12, 14]. Meanwhile, anaerobic microbial activity has been reported to be a factor in the promotion of sludge deflocculation [14]. However, considering the preliminary results in our study, the importance of aerobes and anaerobes on stability of the floc structure might be overstated in literature while the importance of another major group of bacteria, the facultative bacteria, has not been properly evaluated. Facultative bacteria are capable of surviving under both oxygen rich and deficient conditions due to their metabolic capability to respire aerobically and anaerobically [16]. They are widely prevalent in the WWTPs due to the exposure of sludge samples to transient oxygen levels throughout the length of the treatment processes. These

CHAPTER 1

bacteria are also propagated in the cyclically (anoxic-aerobic) cultured SBR sludges. They can also be expected to influence the floc stability and integrity. Their activities could have played a role in the lack of rapid low DO deflocculation in our experimental works. The role of microbes (aerobes, facultative and overall) and their activities on the flocculation process and stability of sludge flocs can be most accurately evaluated only by forcefully inhibiting their activities using a microbial inhibitor [11]. Thus, batch test experiments were conducted by interacting SBR sludge samples with sodium azide, a known mitochondrial uncoupler capable of inhibiting microbial metabolism and activity, under high and low DO conditions. The sodium azide was not studied as a pollutant in these tests, but for its ability to promote microbial inhibitory conditions required to assess the importance of microbial activities on the sludge flocculation. The resulting effects on the floc stability and integrity was assessed. These experiments would elucidate the importance of overall microbial activity and the role of facultative bacteria in mitigating deflocculation under low DO transients.

Along with the microbial communities and their activities, another important factor involved in the maintenance of sludge floc stability are the cations, especially multivalent cations [17, 18]. Studies have reported improved floc formation when multivalent cations were added to sludges. This was due to the ionic interactions propagated between positively charged cations and the negatively charged biological components, especially the extracellular polymeric substances (EPS). Adverse effects on the cationic content, the EPS or their interactions have been reported to promote deflocculation. The SBR sludge samples have been conditioned with divalent calcium and magnesium ions. Their interactions could have played a role in increasing floc

CHAPTER 1

integrity and stability and reduced its propensity to deflocculate under short-term low DO conditions. Thus, batch tests were conducted by interacting the SBR sludge samples with ethylenediaminetetraacetic acid (EDTA), a known chelating agent capable of sequestering divalent cations from the floc body. The subsequent effects of cation chelation on the floc structure would establish the importance of cations and their interactions in the stability of SBR sludge floc ecosystem.

The overall outcomes of this dissertation studies also include the clarification of available knowledge on the biological flocculation process and factors affecting it. This updated knowledge will be vital for the development of more optimal strategies with regards to biological floc maintenance and prevention of deflocculation.

1.3 Thesis Outline

Chapter 1 consists of the introduction to the biological flocculation process and its use in wastewater treatment process. The aims and objectives of the research are also specified along with the significance of the work. This chapter contains part of the review paper which has been published in Chemosphere.

Chapter 2 consists of the literature review, which has been published as a review paper in Chemosphere.

Chapter 3 encompasses the general analytical methods utilized in all the studies, while more study specific methods were mentioned in the respective chapters.

Chapter 4 comprises the results evaluating the impact of DO (high and low DO) on the floc stability and integrity of sludge flocs through batch tests. The supernatant turbidity, EPS quality and quantity and floc size was assessed to establish the

CHAPTER 1

flocculation state in the system. The results have been published as a Technical Paper in ASCE-Journal of Environmental Engineering.

Chapter 5 consists of the results assessing the impact of overall microbial activity on floc integrity, especially under low DO conditions. The sludge samples were inhibited with sodium azide to reduce microbial activity and its subsequent effect on sludge flocculation was evaluated. Microbial activity inhibition was assessed in terms of reduced ammonium conversion and organic carbon removal. The sludge flocculation was evaluated in terms of changes in the supernatant turbidity, EPS and floc size. This chapter has been published in Chemosphere as a Research Manuscript.

Chapter 6 comprises of the results associated with the impact of EDTA addition on the sequestering of cations from the floc body and how this will affect sludge flocculation. The importance of cation chelation on floc integrity will be elucidated by using calcium complexed EDTA and comparing its effects against non-complexed EDTA. This chapter will elucidate the importance of cationic interactions on stabilization of the floc structure. This chapter is being prepared as a research manuscript for submission to a journal.

Chapter 7 consists of the overall conclusions and the practical implications of this study.

CHAPTER 2

CHAPTER 2

Literature Review

Biological sludge flocculation is a complex process that can be influenced by several different factors. Amongst these factors, are the flocs' biological components such as microbial communities therein, and their activities and products (EPS). These are central to the promotion and maintenance of optimal sludge flocculation. Their changes are intricately linked to all the other factors which can significantly affect the state and the direction of biological flocculation. It is from this that the complexity of the biological flocculation arises. Only by having a better understanding of the links between the microbes, EPS and other factors can more effective biological flocculation strategies be developed.

2.1 Floc bio-composition and its role in the biological flocculation process

The most important aspect of the biological flocs are the microbes in the floc ecosystem that aid not only in the floc formation process but also in its consistent maintenance. Some bacterial genera have the ability to secrete extracellular polymeric substances (EPS), which promote adhesion and interactions between microorganisms and the various floc components. These self-aggregating microorganisms are grouped as the floc formers. Another group of microorganisms that have a role in flocculation are the filamentous microorganisms. These provide the frame or backbone of the flocs and contribute to floc strength and stability [19, 20]. A schematic representing a typical floc composition is presented in Figure 2.1 Filamentous microorganisms grow in long thread-like strands, where cells do not separate from each other after cell division and therefore, form filaments. The strongest flocs are considered to be a

CHAPTER 2

balanced mixture of filaments that form a scaffolding for the EPS-producing floc formers to adhere to. Sludge consisting little to no filaments usually produces small and shear sensitive flocs and therefore results in a more turbid effluent. Excessive growth of filamentous species has, however, been attributed to operational problems such as sludge bulking, sludge washout and foaming [19, 21]. Therefore, for well settling flocs, a proper balanced ratio of floc formers and filamentous microorganisms reflected by the filament index is required [22]. The growth of such bacterial combinations can be achieved by adjusting the DO and the substrate concentration accordingly. Very low DO and F/M ratio are known to significantly promote filamentous bacterial growth due to their higher surface area and surface to volume ratio, which allows them to out compete the floc formers [23]. Very high levels of F/M ratio will promote dispersed growth and pin floc formation. Thus, it is essential to establish the optimal growth requirements of both floc formers and filamentous bacteria for a particular sludge and continue to maintain those appropriate balanced conditions for bio-flocculation to be consistent and effective. Protozoa is a common occurrence in activated sludge, representing as much as 5-12% of the mixed liquor dry weight [24, 25]. Protozoans play an important role in regulating the bacterial density as they feed on the planktonic bacteria and dispersed organic particulate matter in the bulk solution [26-28], which impacts the treated water clarity. With regards to biological flocculation, there is some evidence indicating the extensive presence of protozoans under well flocculating conditions [29]. One reported mechanism through which they directly aid flocculation is through the secretion of fecal pellets and mucus [30, 31].

CHAPTER 2

Figure 2.1: A schematic of the floc structure held together by the bound EPS and bacteria.

The balanced microbial population is a key factor in good sludge flocculation. However, microorganisms had been found to constitute only less than 10 % of the organic fraction in activated sludge [10], while the rest was EPS secreted by the microorganisms [32]. EPS is a rich matrix of polysaccharides, proteins, glycoproteins nucleic acids, phospholipids and humic acids [32-34]. The structure and composition of the EPS differed significantly depending on the environments and conditions the microorganisms were exposed to [32]. The EPS surrounding the cells protect them from environmental stresses such as desiccation [10], toxic substances [32] and predatory microorganisms. They could also serve as energy and nutrients sources to neighboring microorganisms in the case of substrate deficient conditions [32]. Most importantly, the EPS components assist in attachment and interactions between microbial cells within the floc helping to maintain floc structural integrity [32, 35, 36]. Among the EPS components, proteins are mainly responsible for imparting

CHAPTER 2

hydrophobicity to EPS [37] and for cation and heavy metal binding [38, 39], which promotes better flocculation and stronger floc structures.

Microbial growth and activities, EPS production and their intra-molecular interactions can be, to a great extent, affected by a variety of chemical and physical parameters, which in turn affect the flocs and biological flocculation as a whole.

2.2 Physico-chemical influence on the bio-components and their subsequent effects on bio-flocculation

2.2.1 Temperature

Temperature variations can influence incoming wastewater properties and subsequent treatment process performance [11, 40, 41]. Temperature is also known to affect the structural and functional properties of the EPS produced by the microbes, for example its viscosity [40], polymer structure, and bacterial cell wall composition [42-44]. Ghanizadeh and Sarrafpour [45] reported decreased bio-flocculation when the temperature was increased from 15°C to 35°C hypothesizing structural sensitivity of EPS at the higher temperature to be the reason. However, they had not supported their conclusions with SMP nor EPS content measurements nor had they considered the possible ionic content and microbial population changes on their results. In another similar study, Krishna and Van Loosdrecht [29] reported excessive extracellular polysaccharides production along with sludge bulking (SVI of 500 mL/g) at temperatures above 30°C. The sludge bulking was attributed to the higher zoogloal and filaments population in the sludge propagated at 35°C [29]. Morgan-Sagastume and Allen [46] reported sludge deflocculation and deterioration of floc physicochemical properties with increase in temperature from 30°C to 45°C. They

CHAPTER 2

concluded the deflocculation occurred due to floc EPS solubilization [46]. In another work, Morgan-Sagastume and Allen [47] reported a net increase in negative surface charge of the flocs with a temperature shift from 30 to 45°C. They hypothesized the temperature change led to the exposed anionic EPS groups [47] and other changes in EPS surface properties, which adversely affected the sludge flocs [34, 46, 47]. Liao, Lin [48] reported the occurrence of filamentous sludge bulking in sludge samples cultured in an SBR at 55°C (thermophilic sludge). This was not the case in SBR sludge samples cultured at 35°C (mesophilic sludge). The differences in EPS composition and the filamentous bacterial content were reported to be possible reasons for the differing bioflocculation behavior of the two sludges [48]. Azimi, Allen [49] also observed increased filamentous bacterial growth with a temperature shift from 12 to 22°C. This led to bigger and stronger but more open-structured flocs. The increased filamentous growth with increasing temperatures is observed to be a common theme with the deterioration of the sludge floc properties in the reported studies [29, 48, 49]. The increased filamentous growth could be caused by lowered oxygen solubility at elevated temperatures if a given plant or reactor had worked at constant aeration rate without residual DO feedback. In addition to the microbial population shift, temperature changes influence the floc properties through the EPS composition [48].

Low temperatures can also adversely affect the sludge samples. Wilén, Lumley [50] observed more fragmented and shear sensitive flocs with higher EPS content during the winter period. At temperatures below 4°C, Wilén, Lund Nielsen [11] observed sludge deflocculation, which was attributed to reduced microbial activity within the flocs. Thus, reduced microbial growth, activity and cell viability reported under low

CHAPTER 2

temperatures are the major reasons cited by different research groups to affect sludge bio-flocculation.

2.2.2 Ionic composition

Apart from its importance in microbial metabolism, pH governs the dissociation of functional groups in EPS such as carboxylic acids, which generally remain deprotonated at pH above 4.0, while groups like thiol or amino become electro-neutral at higher pH [52-55]. The higher the number of dissociated groups, the higher the polarity and therefore hydrophilicity of the biomass. The EPS produced are dependent on the microbial communities in the sludge and the conditions to which each sludge is subjected. Therefore, a sludge's flocculating ability, although following similar general trends with respect to pH changes, may differ where the optimal pHs are. It has been shown for example, that flocculating activity of *Bacillus* sp. PY-90 was high in the 3-5 pH range [56] while the maximum activities of *Enterobacter* sp. BY-29 and *Streptomyces griseus* were observed at pH 3 [57] and pH 4 respectively [58]. *R. erythropolis*, *Aspergillus* sp. JS-42 and *Paceilomyces* sp. were reported to be active at neutral pH [59, 60], while the activity of *A. sojae* increased when the pH exceeded 7 [61, 62]. pH is also known to affect the EPS production itself [63, 64]. This was the case with *S. thermophilus* strains producing different amounts of exopolysaccharides depending on the pH at which it was cultured [65]. In certain cases, the pH required for growth can be different from the pH required for a metabolic activity or EPS production [66]. Thus, consideration of a suitable pH is dependent on several different factors including microbial community, the type of EPS required and the application for which it is required (such as for biological flocculation) [55, 63].

CHAPTER 2

Figure 2.2: Major phenomena governing EPS interactions: a) dissociation of functional groups (adapted from [67], b) repulsive electrostatic vs. attractive Van der Waal forces and c) multivalent cation bridging theory (adapted from [68, 69]).

Within pH ranges typical for activated sludge process operation, the collective dissociation state of functional groups in the EPS would usually result in overall negative charge accumulation and electrostatic repulsion of EPS chains. The most important phenomena governing EPS interactions are schematically presented in Figure 2.2. Cations present in the solution are believed to aid in charge neutralization [70-78]. Nguyen, Hilal [79] concluded that sludge flocculation was mainly driven by cation (Ca^{2+} and Al^{3+})-biopolymer interactions and polymer gelation [79]. Park, Muller [80] observed that effluent quality and digestibility of sludge from different wastewater treatment plants strongly depended on the major influent cations such as Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Fe^{3+} and Al^{3+} . Bruus, Nielsen [73] demonstrated sodium at high concentrations can displace calcium from the floc, and lead to deflocculation (increased supernatant turbidity and specific resistance to filtration). Tezuka [72] also reported the importance of calcium and magnesium in the role of bio-flocculation process for monocultures studies. According to the divalent cation bridging theory, multivalent cations such as Mg^{2+} , Ca^{2+} and Fe^{3+} [77, 81, 82] are expected to bridge

CHAPTER 2

negatively charged functional groups within the EPS and contribute to more pronounced bio-flocculation [77, 81, 82]. Higgins and Novak [74] came up with a theory that deflocculation can be triggered when the ratio of the sum of the monovalent cation concentrations to the sum of the divalent cation concentrations is more than 2. The results were confirmed by Sobeck and Higgins [68] and the authors concluded that the discrepancies between different studies were mainly due to differences in experimental methods used, especially the short-term batch tests versus continuous flow reactor studies. Eriksson and Alm [70] reported the floc formation to be dependent on polyvalent cation interactions, which they confirmed by complexing calcium ions with a chelating agent (ethylenediaminetetraacetic acid, EDTA) resulting in reduced floc formation. Biggs, Ford [83] reported that calcium is an effective flocculant even after prior flocs dispersal by sonication. However, Bruus, Nielsen [73] reported the exchange of calcium with magnesium ions also caused a deterioration in floc properties due to the higher specificity of EPS to Ca^{2+} . In another study, Arabi and Nakhla [84] found increased magnesium content was beneficial in reducing membrane biofouling by promoting bigger flocs with higher content of high molecular weight EPS along with reduced hydrophobic SMP depositions [84]. Flocculation is influenced not just by the valency of the interacting cation but also by the long-term sludge conditioning. Such cation conditioned sludges will react differently from unconditioned sludges to changes in cation addition. Moreover, other properties like the hydrophilicity of cations, its specificity to EPS can influence its interactions with the EPS and subsequently the floc integrity. The influence of cation on EPS is also observed in granulation where bacteria EPS interaction stabilized the microstructure [85, 86]. Jiang, Tay [85] reported that Ca^{2+} augmentation halved the time necessary for

CHAPTER 2

sludge granulation from 32 to 16 days, and granules fed with calcium rich feed showed better settling and had higher polysaccharide contents. Li, Liu [86] reported similar reduction of granulation time from 32 days to 18 days by dosing Mg^{2+} instead. It has to be specified that the sludge in this case was conditioned with Mg^{2+} from the beginning, indicating the significant effect of granule conditioning on its interaction with multivalent cations and sludge settling [73, 86]. Ren, Liu [87] reported calcium-rich granules were more structurally rigid and stronger due to $CaCO_3$ formation, which acted as nucleus for the formation of granules and EPS- Ca^{2+} interactions.

Trivalent cations such as iron or aluminum have been frequently used to improve settling characteristics in activated sludge treatment plants and in promoting better bioflocculation. This is due to its high charge valence and polarizability and relatively smaller degree of hydration [18, 79, 80]. According to reports, the flocculation of activated sludge into pelletized form has higher correlation to trivalent cations concentration than to divalent and monovalent cations [17, 18]. The trivalent cations were almost entirely concentrated in the floc pellets bound tightly to the EPS unlike the bivalent and monovalent cations. They also noted that anaerobic sludge could bind to more trivalent cations over time, and thus sludge flocculation improved with longer SRTs. Agridiotis, Forster [88] reported $FeSO_4$ treatment reduced the stirred sludge volume index (SSVI) of a filamentous and hydrophobic sludge (300 to 90) and made flocs more compact in structure. When $AlCl_3$ was used, the settlement index was initially improved, but a deterioration of the sludge properties was observed later [88]. A reason postulated was that the deterioration could be caused by charge reversal on the floc surface due to excess of aluminum addition. However, this was not the case

CHAPTER 2

with iron addition and thus the authors concluded that floc deterioration during aluminum dosing was due to more complex reasons without further elaboration [88]. Paris, Lind [89] observed that during the AlCl_3 dosing to activated sludge, the hydrophobicity and floating tendency of the flocs were decreased. They postulated it could be due to the interaction of aluminum species with lipophilic substances and the hydrophobic surface of the filamentous *M. parvicella*. Caccavo, Frolund [90] reported the changes in Fe^{2+} concentration in activated sludge had no clear effect on sludge flocculation while decreasing concentrations of Fe^{3+} ions resulted in higher supernatant turbidity. They also observed the reduction of Fe^{3+} in sludge flocs by iron reducing bacteria resulted in higher supernatant turbidity. Nielsen and Keiding [91] found the addition of sulfide to activated sludge weakened the floc strength and increased shear sensitivity. They suggested the specific reduction of Fe^{3+} to FeS to be the cause of the deflocculation phenomenon since the same results were not observed for sulphide spiked sludge samples not supplemented with Fe^{3+} . In addition to the beneficial effects of iron and aluminum on flocculation as multivalent cations, its toxic effects should also be considered, and this is discussed more in the “Toxins” section.

2.2.3 Toxins

Biological wastewater treatment systems are highly susceptible to shock loads of toxic chemicals. Microorganisms are known to activate stress responses in the form of increased EPS production to protect themselves from damage by binding the toxins before they reach the microbial cells [92].

Heavy metals like Hg^{2+} and Cr (VI) have been reported to adversely affect the microbial communities, their activities and subsequently the floc structural (floc size

CHAPTER 2

decrease, pin floc formation) and functional properties [30]. The microbes counteract this heavy metal toxin stress partially through the increased production of EPS [93, 94] that have high capacity for heavy metals adsorption [63, 95-97]. Studies have shown the carboxylic or phosphoric groups present in EPS promote the complexation of heavy metals like Hg^{2+} [96, 98-100] and the affinity of EPS to bind Hg^{2+} is stronger than its affinity for Ca^{2+} interaction according to Sheng, Xu [101]. However, shock loads can overcome EPS adsorption to promote irreversible damage to microbial communities, their activities, EPS and the biological floc matrix [30]. Nanomaterials are another growing concern with regards to their toxic effects on biological wastewater treatment processes [102-104]. The toxicity and floc destabilizing effects of nanomaterials are directly proportional to the concentration and time of exposure to the sludge flocs [105-107]. Reported studies speculate the increased surface charge, cell lysis and decreased hydrophobicity as reasons for the floc destabilizing effects and reduced settleability [106, 107]. Similar to heavy metal toxicity, the microbes have been reported to counteract this stress through the increased production of EPS, especially LB EPS, to adsorb the toxins but this counter effectively reduces the floc strength [108]. Other toxic compounds that can affect the sludge floc stability include pharmaceuticals and surfactants [109-116]. Studies have shown the long-time exposure of aerobic granules with fluoroquinolones like ciprofloxacin, ofloxacin and norfloxacin did lead to adverse effects like reduced granule size and increased supernatant turbidity which are hallmarks of granule degradation. Recovery only occurred when the fluoroquinolones were removed from the system indicating their toxicity [109]. Sodium dodecyl sulfate spiking was reported to result in increased negative charge on sludge EPS and this led to pronounced deflocculation and

CHAPTER 2

reduction in flocculation efficiency [117]. Dodecyl dimethyl benzyl ammonium chloride (DDBAC) was reported to promote solubilization of TB EPS and increase in LB EPS [118] and this can be expected to destabilize the floc matrix. One must also bear in mind that anionic surfactants are strong protein denaturants, which when interacted with the bound protein content in the flocs can be expected to promote floc destabilization and even deflocculation. Their interactions with lipid-membrane proteins may also lead to cell lysis [119].

Despite the advantages offered with regards to improved flocculation and settling, considerations have to be made for the possible toxic effects of Al^{3+} and Fe^{3+} . Al^{3+} toxicity is known to severely affect humans, animals and environment under certain conditions and concentrations [120]. In one study, aluminium and iron toxicity was reported on a rotifer, *Lecane inermis*, widely observed in activated sludge when added as aluminum chloride and ferric chloride salts [121]. Iron toxicity in cells, through the generation of reactive oxygen species (superoxide and hydroxyl radicals) capable of damage at the DNA level, has been reported by De Freitas and Meneghini [122]. Philips, Rabaey [123] reported progressive inhibition of microbial activity upon continuous dosing of either ferrous or ferric iron salts to activated sludge, with the latter having more persistent effect. The observed toxicity was speculated to be due to the pH drop caused by the formation of iron hydroxides and nitric oxide, a bacteriostatic agent, generated during the reaction between ferrous and nitrite/nitrate. The generation of ROS is another possible reason for the increased toxicity that the authors speculated on, but no study was done to verify this [123]. The toxicity levels have been shown to depend on the sludge, the microbes and other conditions. Thus,

CHAPTER 2

the several advantages provided by Al^{3+} and Fe^{3+} addition must be carefully balanced with their possible toxicity and adverse effects on the sludge. This way, their beneficial effects in terms of promoting stronger ionic interactions can be maximized.

2.3 Influence of process parameters on the sludge flocs and biological flocculation

It is hard to avoid adverse conditions when treating wastewater, composition and physical parameters of which are not consistent over time. However, it is still possible to achieve a level of control by adjusting the changeable operating conditions in WWTP, which enables the development of suitable flocculation control strategies. Optimal process conditions for ensuring proper biological flocculation are those geared towards the promotion of the right balance of microbial communities and EPS quality and quantity. The most influential process parameters are discussed below.

2.3.1 Dissolved oxygen

Aeration of the aerobic tank is one of the primary operating tools enabling control over sludge flocculation. Extremes of DO can bring an adverse effect on flocs size and sludge settling. Low DO and anaerobic conditions bring about reduced activity of aerobes and excessive filamentous growth [64, 129], while high DO conditions would accelerate the decomposition of excreted EPS [64]. Flocs in sludge studied by Wilén and Balmér [129] were observed to be most compact, stronger and better settling when the DO concentration was maintained between 2-5 mg/L. In another work, Wilén [130] reported poorer effluent quality and sludge qualities in a full scale treatment plant at a lower DO concentration of 2 mg/L when compared to its conditioning with 4 mgDO/L. Excessive filamentous growth is an issue when the DO is very low (1 mg/L or below) and this was also cited in reports to be the reason for the porous and more

CHAPTER 2

irregularly shaped flocs along with a higher SVI [129, 130]. Zhang, Zhang [64] reported the coagulating capacity of EPS produced by activated sludge subjected to a DO of around 4 mg/L was changing over time within the performed 12-hour experiment. During the experiment, EPS was frequently extracted and used for coagulation of a kaolin suspension. The most efficient kaolin flocculation was observed for EPS sample extracted from sludge at two hours of aerobic treatment. They concluded it was due to the highest yield of EPS production at that time rather than any other reason. This was because the FT-IR spectra of EPS and components ratio (polysaccharides-30%, proteins-50% and nucleic acid-20%) produced before and after aerobic digestion were almost the same, with only the total concentration differing between the two [64]. In another experiment, they compared the flocculating ability of the same sludge after subjecting it to low DO conditions of below 1 mg/L and observed a gradual decrease in the flocculating abilities of the extracted EPS [64]. One consideration that has to be made is the fact that Bradford method was used for protein measurement [64]. Polyphenol contamination can affect the protein measurement by Bradford method and the humic acid in the sludge samples could have been read as proteins using this method [131, 132]. Using another method like Lowry's modified method can distinguish the color developed from protein and humic acid [10, 133]. Making this change in the analytical method could have yielded a more accurate view on the impact of EPS components on the flocculation result observed.

Wilén, Keiding [14] observed increased turbidity and soluble EPS content in the supernatant within 2 hours of severe DO limitation. Anaerobic respiration, metabolism and reduced aerobic microbial activity were speculated to be reasons for the observed deflocculation. This was partly exhibited by the addition of *Shewanella* sp., a

CHAPTER 2

facultative bacterium, whose addition under anaerobic conditions resulted in even more deflocculation. Re-aeration of the deflocculated samples was shown to result in re-flocculation, in terms of reduced supernatant turbidity. The re-flocculation upon re-aeration was reported to be rapid, but not complete indicating some deflocculative changes were irreversible. It has to be considered that the WWTP sludges used in their experiments were not re-fed before the start of the batch test [14]. Starvation effects could have also been partly responsible for the deflocculation results observed [14]. The floc size changes were also not monitored in this study, which reduces the impact of the turbidity results and the speculation of deflocculation. The presence of nitrate as an electron acceptor has been reported by various groups to mitigate the deflocculation under low DO conditions [14, 123]. The usage of nitrate for anoxic respiration hints at the activity of facultative bacteria and not just strict aerobes, which was not signified by Wilén, Keiding [14] in their speculation on the mitigation of deflocculation.

Oxygen concentration is also important for redox processes that may occur during the treatment. The best-known example is oxidation and reduction of iron present in the sludge in the form of complexed ions or precipitated hydroxides/oxides. In a study where the oxidation-reduction potential (ORP) was as low as -400 mV (typical for anaerobic conditions) in an anoxic reactor, the addition of iron in the form of FeCl_2 resulted in increased SMP protein content while the EPS protein content was reduced [134]. This is symptomatic of deflocculation as per reported literature [15]. In the same study, when FeCl_2 was dosed to an aerobic/anoxic reactor maintained at an ORP of +30 to +80 mV, the EPS protein content was increased while the SMP protein content was reduced [134]. Wilén, Lund Nielsen [11] hypothesized the reduction of Fe(III) to Fe(II) by the addition of *Shewanella* sp., a facultative anaerobic bacterium,

CHAPTER 2

resulted in increased supernatant turbidity in the sludge samples. These reports indicate the oxido-reduction of iron and other ions could significantly influence floc stability and integrity through their interactions with the other floc components, which further signifies the effect of DO levels on the biological flocculation process [64, 91, 134].

One has to bear in mind the DO level in the bulk of the reactor does not reflect the actual conditions inside of a floc. The dissolved oxygen (DO) concentration distribution within a floc has been hypothesized to affect its microbial population distribution [135-137], with the aerobic microbes on the outside of the flocs metabolizing and providing the substrates for the anaerobic microbial community in the floc core. The size of a floc and viscosity of the produced EPS are of a great importance for the sludge performance and the same DO levels in two different reactors may lead to sludges of very different flocculating abilities.

2.3.2 Feeding regimes

The F/M ratio is an important parameter which can influence biological floc formation and maintenance. At low F/M ratio, the propensity to form biological flocs is higher in order to maximize the efficiency of substrate utilization [138]. Remedial action for the mitigation of dispersed growth problem is a reduction in the F/M ratio of the system, by raising the biomass concentration. At fixed loading rate, adjustments to the F/M ratio can be done through changes to the solids retention time (SRT), which affects the biomass concentration [139]. The optimal F/M ratio is observed to be specific to the sludge and the types of microbial communities in the sludge. It is vital to establish the optimal F/M ratio range for the sludge, which will promote the growth of both floc

CHAPTER 2

forming and filamentous bacteria required for effective floc formation and maintenance. Along with the F/M ratio, feeding patterns (fast feeding vs slow feeding) and organic loading rates have been reported by several studies to also influence the floc structural development and stability through their impact on the types of microbes communities cultivated and their active metabolism [50, 138, 141-148].

Feast and famine feeding regimes have also been reported to play an important role in effective sludge flocculation and granulation [150, 151]. A good floc is a balanced combination of filaments and floc-formers. However, they have different growth kinetics which necessitates the need for specialized feeding regimes [23, 152, 153]. The feast-famine cycling of feeding conditions can theoretically promote conditions satisfying the growth and metabolism of both floc-formers (high substrate utilization rate and growth rate) and filaments (lower growth rate but lower decay rate) [153]. The other advantage of this feeding regime is that the induced conditions will lead to the enrichment of slow growing bacteria capable of accumulating and utilizing storage polymers [150, 154] as required along with increased EPS production, especially under SBR configuration as reported by Ehlers, Wagachchi [151]. This can then lead to the formation and maintenance of stronger and more compact flocs and granules, as reported by Wilén, Liébana [155] based on their literature survey.

2.3.3 Solids retention time

Solids Retention Time (SRT) represents the average duration the sludge is retained in the system. This will affect the amount of food available for microbial consumption and process parameters such as reaction rates, oxygen demand and effluent quality. The selection and control of microbial communities in the sludge are performed

CHAPTER 2

through adjustments to the SRT [156], which in turn will significantly influence sludge flocculation. Recent reports have even reported the influence of SRT on factors like the type of EPS produced [156, 157] and the ionic interaction with the floc EPS [18].

Longer SRT (around 15 to 20 days) has been reported by multiple studies to benefit sludge flocculation. Considering the reports, the optimal SRT to ensure proper flocculation was rather a relative value which ensured the following: promoting proper microbial growth and activity [139], providing the time for promoting interactions between EPS and cations [18] and avoiding the prevalence of endogenous respiration due to substrate and nutrient depletion [140]. The sludge flocs were richer in bound EPS concentration. The increased bound EPS content improved interaction and adhesion among the floc components, resulting in larger, stronger and more compact stress resistant flocs [47, 142, 158-160]. The optimal SRT was also found to have lowered the interaction energy between floc particles, led to a reduction in LB EPS concentration and an increase in the multivalent cation-EPS interactions. This promoted an improvement in sludge settleability along with a reduction in SVI and effluent turbidity [18, 156, 157].

However, some contrary results have also been reported with regards to the impact of SRT on the flocculation process. Scott, Liss [161] reported that EPS composition (protein, carbohydrate, and DNA content) of flocs were not significantly affected by varying the SRT from 4 to 20 days. Azimi, Allen [49] reported that the mechanical strength of the flocs, in terms of median shear stress value, turbidity and effluent suspended solids concentration was not significantly different between flocs cultivated at an SRT of 7 and an SRT of 20 days. In their study, they reported the temperature at

CHAPTER 2

which the reactors were operated had a more profound influence on the flocs than the SRT.

2.3.4 Right nutrients balance

The availability, abundance and composition of consumable substrates (“food”) are widely known to have a major effect on the sludge properties and performance, and this includes the sludge flocs. It is common knowledge microorganisms require a variety of other nutrients along with a carbon source, including nitrogen and phosphorous sources, for their growth and viability. Traditionally, a C:N:P ratio of 100:5:1 is regarded as the minimum nutrient requirement of activated sludge systems designed for carbon removal [162]. Erkan, Engin [163] observed an increase in C/N ratio favored synthesis of EPS carbohydrates and a reduction in EPS proteins synthesis and sludge hydrophobicity. Durmaz and Sanin [164] observed similar results in terms of EPS content and hydrophobicity. The changes in EPS composition can then be expected to influence floc structure and properties accordingly. Ye, Ye [165] observed when C/N ratios were changed from an optimal value of 20 to either 4 or 100, the floc characteristics were adversely affected resulting in increased supernatant turbidity. The LB EPS content alone changed with the C/N ratios, and this could have reduced the floc strength and made the flocs more sensitive to deflocculative stresses, which resulted in the increased supernatant turbidity. Miqueleto, Dolosic [166] reported increased EPS production at higher C/N ratios (13.6 gC/gN) with glucose and urea acting as the carbon and nitrogen source. This was considered to be through the activation of particular metabolic pathways by the bacteria under those conditions [166]. However, WWTP influent wastewaters will be much more complex than the

CHAPTER 2

simpler synthetic wastewaters used in laboratory research. The microbial consumption of these organics for their growth, metabolism and flocculation activity can be expected to be different in the treatment settings. The optimal C/N ratio for effective biological flocculation will thus have to be carefully considered on a case by case basis depending on the incoming wastewater, the microbial communities in the sludge and the treatment process requirements.

With regards to the phosphorus content in the feed, Ericsson and Eriksson [167] reported the extreme pre-precipitation of the phosphorus content resulted in decreased sludge settleability along with an increase in SVI and filamentous growth. Liu and Liss [168] on the other hand, reported an increase in gravitational settling velocity, compactness and size of flocs at phosphorous content as low as 100:5:0.1 (C:N:P). Azimi, Allen [49] reported the flocs cultivated under P-limited conditions (C:N:P = 100:10:0.3) to be stronger than the flocs grown under P-normal conditions (C:N:P = 100:10:1) or P-starving conditions (C:N:P = 100:10:0.03). This indicates the phosphorus can be limited to a certain limit before the flocs are adversely affected [49]. Also the source of nutrients has a significant impact on flocculation as significant changes have been reported in the internal and surface properties of microbial flocs under conditions of various carbon and nitrogen source [157, 169-173].

The complexity of the biological flocculation process is such that the complex interplay between the floc bio-components and other factors must be recognized and importance must be given to adapt to various changes in the system. The influence of these various factors in the treatment system on each other and the biological flocculation process has been summarized in Table 2.1.

CHAPTER 2

Table 2.1: Interconnected factors influencing biological sludge flocculation

Factor	Impact on flocculation
Temperature (High/Low)	<p>Influence on flocculation: Microbial growth and metabolism, EPS structural integrity and interactions, DO content, pH and ionic interactions</p> <p>Influenced by: Climate, influent conditions, treatment process conditions, use of temperature control</p>
Ionic composition (pH/monovalent/polyvalent cations)	<p>Influence on flocculation: Microbial growth and metabolism, EPS structure and interactions, EPS-cation interactions (cation bridging)</p> <p>Influenced by: Temperature (influencing pH), influent ionic composition, addition of ions during treatment, inherent ionic content of the sludge (for e.g. bound to sludge flocs)</p>
Toxins (Heavy metals/nanoparticles/pharmaceuticals/surfactants)	<p>Influence on flocculation: Microbial growth and metabolism inhibition, adverse effects on EPS structure and integrity, weakened EPS interactions, different adverse effects based on toxin type</p> <p>Influenced by: Influent toxin content and concentration, treatment and process conditions, microbial and EPS-toxin interactions</p>

CHAPTER 2

Process Parameters	
Dissolved oxygen (High/Low)	Influence on flocculation: Microbial growth and metabolism, microbial community selection, microbial activity, EPS production (quantity and quality), oxido-reduction reactions (iron) Influenced by: Temperature, microbial activities, aeration intensity
Feeding regimes (optimal feed requirements for each sludge depend on the process outcomes)	Influence on flocculation: Microbial growth type (planktonic or flocculant), microbial community selection, microbial activity, EPS production (quantity and quality) Influenced by: SRT, sludge VSS
Nutrients (widely utilized optimal C:N:P ratio is 100:5:1 for aerobic treatment processes)	Influence on flocculation: Microbial growth, microbial community selection, microbial activity, EPS production (quantity and quality) Influenced by: Influent constituents, pre-treatment steps

2.4 Indicators of flocculation and deflocculation

Control, improvements and optimization of biological sludge flocculation should be accompanied with constant or regular monitoring to establish the suitability of the control and mitigation strategies implemented. For this purpose, there are

CHAPTER 2

characteristics and indicators that can be very informative of a system's flocculation condition. Improvements in flocculation can normally be correlated to improved floc structural characteristics (direct) and liquid-solids separation (indirect). Structural characteristics include floc strength, density, structure and size. Improvements in flocculation will usually be accompanied by an increase in the floc resistance to stresses, increased compactness and density leading to better settling flocs. Thus, using a combination of direct (floc density, strength) and indirect (settleability, floc size changes, turbidity) measures, the flocculation condition in the system can be established more accurately.

Sludge volume index (SVI) is the most common measure of sludge settleability and compactness used by WWTP operators, because it is an easy and fast method to determine the performance of secondary clarifiers. The test consists of settling 1 L of sludge in a graduated cylinder and reading the volume of settled sludge after 30 minutes. The final volume of the settled sludge, divided by the sludge total suspended solids (TSS) concentration, gives the SVI in ml/gSS. The SVI test is sometimes complemented by the initial settling rate (ISR) measurements, which describe the maximum velocity of particle settling at the beginning of the process.

Particle size distribution (PSD) is a measurement of floc sizes and the degree of sludge flocculation. It does not directly measure the settling characteristics of sludge or the flocculation behaviour, but it provides valuable information to predict the sludge settling behaviour and floc characteristics. Reduction in particle size followed by an increase in suspended solid concentration could be an indication of floc break-up due to some kind of a stress. However, particle size changes alone cannot be construed as

CHAPTER 2

an indicator of a deflocculation event and must always be considered together with the other data to come to accurate conclusions. PSD is determined by light scattering techniques using lasers and visible light, or by direct microscopic observation and measurements of flocs [177].

Floc strength measurement determines the stability of the activated sludge floc under shear forces. Many methods were developed for floc strength measurement, both at the macroscopic and microscopic (single floc) levels [178]. A popular macroscopic method is the standardized shear reactor test. Sludge is stirred in a standardized baffled reactor and the agitation speed is manipulated to control the shear force exposure. After stirring, sludge properties such as particle size distribution and supernatant turbidity were measured for determination of floc strength [179, 180]. Although floc strength measured with different techniques cannot be directly compared, these measurements can be used to assess the effect of different conditions on activated sludge floc strength and integrity [178]. Higher floc strength can be linked to larger and more stable flocs, and this can be mostly related to faster sludge settling and better liquid-solids separation [177, 181].

Increased supernatant turbidity along with an increase in TSS concentration can indicate floc breakage or deflocculation. These indicators have been used in several studies to assess flocculation [11, 12, 14], however, this alone cannot confirm the occurrence of deflocculation. The wastewater treatment system contains planktonic bacteria and microbes as well, i.e. free-swimming microbes not associated with flocs. The changes in supernatant turbidity can also be a result of these bacteria, which might provide inaccurate evaluations of the biological flocs in the system. It is thus essential

CHAPTER 2

to monitor the floc size changes along with the supernatant turbidity and soluble microbial product levels to validate the occurrence and type of deflocculation (surface erosion or floc fragmentation).

The quantity and composition of associated EPS are also good indicators of sludge flocculation. The SMP concentration can be utilized to assess the integrity of floc structures and the degree of flocculation. In addition to the SMP, monitoring of the bound EPS content can also be used to assess the floc structure and flocculation performance. Increased amount of LB-EPS was observed in various studies to adversely impact sludge flocculation (floc structure and integrity) and liquid-solids separation [157]. A reason for this was the poor cell attachment under these conditions. The LB EPS content has also been reported to be observed in increased concentration under toxic conditions. The increased LB EPS interact with and reduce the toxicity of heavy metals and toxins [108]. Excess EPS carbohydrates, associated with high C/N/P ratio or deficiency of other nutrients or oxygen, can lead to “slime bulking”, while insufficient EPS production resulting from prolonged starvation can result in floc erosion or pin flocs. Regular monitoring of the unbound and bound EPS levels can be indicative of the changes occurring in the biological flocs, which can then be used to optimize the treatment conditions to help maintain the sludge flocs and mitigate deflocculation.

2.5 Summary and Research Gaps

The following conclusions were drawn from the literature review:

CHAPTER 2

- Microbial composition of sludge has a significant effect on its flocculating abilities, which are specific for a given sludge, the influent and treatment conditions.
- Microbial EPS, in the right quantity and quality, is required to promote effective flocculation and this must be considered more in flocculation measures.
- Many chemical substances can affect the properties of the produced EPS. Some of them, like multivalent cations, help flocculation by linking EPS polymers, tightening and strengthening the structure of a floc. Some, can cause disruption of EPS interactions and lead to deflocculation, like in the case of toxins like anionic surfactants. Finally, some substances can adsorb on the surface of EPS increasing compactness of the floc, which in turn may result in insufficient metabolites diffusion within its structure.
- A variety of control handles are available to ensure proper microbial activity and biological flocculation. Of these the most basic and most effective measures are the DO levels, SRT and the feeding regimes. Comprehensive and consistent characterization and monitoring of the building blocks (microorganisms and EPS) of activated sludge will allow evaluation of the flocculating abilities of a sludge as well as prediction of direction in which bioflocculation will evolve if given physical or/and chemical conditions occur.

Maintaining good condition of analytical tools is of huge importance for this.

From the literature review, some inconsistencies were observed mainly with regards to the observation of deflocculation under short-term low DO transients or due to other deflocculative stresses. The reported studies have mostly defined the occurrence of

CHAPTER 2

deflocculation based on increasing supernatant turbidity and SMPs in the tested sludge. It has to be stressed that sludge samples contain free-swimming planktonic microbes in addition to the floccular microbial communities. These planktonic microbes are more sensitive to sudden changes in conditions, which can cause their death and cell lysis. The disruptions to the planktonic bacteria, especially in a short timeframe, can contribute to the regular symptoms of deflocculation like increased supernatant turbidity and SMPs while masking the real effects on the biological flocs. The negative impact in this case can be temporary with the flocculation recovered by removing the respective stress on the planktonic microbial communities. Deflocculation can be properly established only by monitoring the floc size changes, and possibly changes in the bound EPS content. This is because the floc size and bound EPS reduction can have more long-lasting impacts on the sludge settleability and liquid-solids separation process. It is thus essential to reconsider and reevaluate the deflocculation phenomena while considering the holistic impact on the flocs themselves.

Another aspect of the deflocculation phenomena that needs to be clarified is the role of certain group of microbial communities in the maintenance of sludge flocs. Aerobes and their activities, including EPS production, are reported to help with floc maintenance, while anaerobic microbial activities are reported to promote deflocculation. However, no consideration has been given for facultative bacteria. These bacteria can metabolize and grow under low DO conditions, which can help maintain the floc stability under these conditions and prevent deflocculation. Their role in the biological flocculation process should be clarified, especially since they are more prevalent in WWTPs and cyclic aerobic-anoxic conditioned sludges.

CHAPTER 2

Many of the reported studies on biological sludge flocculation processes have concluded their results in absolute terms and had not considered the effect of relativity since every sludge is different and will react to conditions and process parameters differently. This relativity can be expected to significantly influence a sludge flocs' reaction to short-term transients in conditions, i.e. low DO transients or toxin spike. Thus, this must be properly evaluated to establish the importance of analyzing complex biological systems, like flocs, in relative terms for better understanding of their workings and the development of more optimal flocculation strategies.

CHAPTER 3

CHAPTER 3

Analytical Methods

This chapter encompasses the analytical methods utilized across the entire study. More specific methods will be mentioned in the respective chapters.

3.1 Total suspended solids (TSS) and volatile suspended solids (VSS) measurements

Total suspended solids (TSS) and volatile suspended solids (VSS) measurements were performed in accordance with Standard Methods 2540 (APHA 2005) [182].

3.2 Dissolved oxygen carbon (DOC) and soluble chemical oxygen demand (COD) measurements

The samples were filtered with a 0.45 µm pore size filter prior to DOC and soluble COD measurements. The DOC measurements were performed using a TOC/TN analyser (TOC-L/TNM-L, Shimadzu, Japan). The soluble COD measurements were performed with Hach Test Kits (High Range/Low Range/Ultra-low range) and Hach DR1900 Spectrophotometer (Hach, USA).

3.3 DO and ionic measurements

DO and pH were monitored with a benchtop DO meter (YSI 5100, USA) and a pH meter (Orion Star A111, Thermo Scientific, USA), respectively. Potassium and ammonium concentrations were measured simultaneously with potassium (Orion 9319BN, Thermo Scientific, USA) and ammonium-selective electrodes (Orion 931801, Thermo Scientific, USA) against a double-junction reference electrode (Orion 900200, Thermo Scientific, US with 0.1 M NaCl as outer chamber filling solution), using a 16-channel EMF interface (Lawson Laboratories, Inc., Malvern, PA, USA).

CHAPTER 3

3.4 Turbidity measurements

The sludge samples were left undisturbed and allowed to settle for 10 minutes. The resulting supernatant was collected, and the turbidity was measured with a double beam UV-Vis Spectrophotometer (UV-1800, Shimadzu, Japan) or Turbidimeter (Hach 2100N, USA). The readings were expressed in terms of absorbance at 650 nm or Nephelometric Turbidity Unit (NTU) respectively.

3.5 Floc size measurements

The floc size of samples was measured with a Laser Diffraction Particle Size Analyzer (SALD 3101, Shimadzu, Japan). The sample volumes added were such that the absorbance values were identical across all of the tested samples. For floc size measurements, sonication was turned off while the stirring speed was reduced from a maximum of 525 rpm (setting 6) to 315 rpm (setting 4) to minimize the effects of shear stress on the flocs. The pump speed was also reduced from setting 6 (5500 cm³/min) to setting 4 (3000 cm³/min) for the same purpose. Each sample was measured 5 to 8 times over a 2-minute period from which mean values were calculated and reported.

3.6 SMP & bound EPS extraction protocol

The SMP and bound EPS were extracted by a variation of the mild heat extraction method [157, 183]. Briefly, supernatant was collected from 20 mL of sludge samples after settling for 10 mins and filtered through 0.45 µm pore size filter paper. The filtrate was considered as containing the soluble microbial products (SMP) fraction. Meanwhile, the settled cell pellet was first centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 6,000 g for 10 minutes before the pellet was resuspended to the initial

CHAPTER 3

volume (20 mL) with pre-heated (52°C) 0.05% w/v NaCl solution. The sample was agitated at 37 KHz in an ultrasonication bath for 2 minutes and then centrifuged at 8,000 g for 10 minutes. The supernatant was filtered with a 0.45 µm pore size filter and the filtrate was then considered to hold the loosely bound (LB EPS) fraction. The pellet was again resuspended to the initial volume with pre-heated (52°C) 0.05% (w/v) NaCl solution, and then incubated at 52°C for 1.5 hours in a water bath (Waterbath WNB 10, Memmert, Germany). After incubation, the samples were centrifuged at 11,000 g for 10 minutes. The supernatant was filtered with a 0.45 µm filter and the filtrate was considered to hold the tightly bound (TB EPS) fraction. The samples were stored at 4°C till further analysis.

3.7 Nitrate and nitrite measurements

The nitrate measurements were performed with Hach test kits (TNT 836 and 835 kits), while the nitrite measurements were performed using the FIA analyser (QuikChem 8500 Series, Lachat Instruments, USA) with a high throughput method [184]. The measurements were performed with samples filtered through a 0.45 µm pore size filter.

3.8 Colorimetric measurements

The protein and humic acid contents in the filtered sludge samples were measured according to the modified Lowry's method [133] while the phenol-sulphuric acid method was used to measure the carbohydrate content [133, 185].

3.9 FEEM and synchronous fluorescence analysis

The fluorescence excitation emission matrix (FEEM) has been utilized to monitor the soluble microbial product composition through their unique fluorescence profiles.

CHAPTER 3

Different regions in an FEEM can be designated to a particular fluorophore based on their particular Excitation (Ex)/Emission (Em) wavelength maxima regions (Table 3.1) [186, 187]. The fluorescence corresponding to the EPS components could then be quantified using the fluorescence regional integration of the fluorescent intensities in their specified regions [186, 187].

Table 3.1: The FEEM regions corresponding to the fluorescing EPS components (adapted from [186])

FEEM Region	Fluorophore
Region I	Tyrosine/tryptophan amino acid-like with Ex = 220-240 nm and Em = 280-360 nm
Region II	Tyrosine/tryptophan protein-like with Ex = 220-295 nm and Em = 280-360 nm
Region III	Polysaccharide-like or microbial product-like with Ex = 300-330 nm and Em = 360-390 nm
Region IV	Fulvic acid-like with Ex=220-240 nm and Em =410-450 nm
Region V	Polyaromatic humic acid-like with Ex = 260-290 nm and Em =420-460 nm
Region VI	Polycarboxylate humic acid-like with Ex = 330-370nm and Em = 420-460 nm

CHAPTER 3

Figure 3.1: The various fluorophore regions in the FEEM contour graph (adapted from [186]).

The fluorescence excitation emission matrix (FEEM) was performed using a Cary Eclipse Fluorescence Spectrophotometer (Agilent, USA). The excitation and emission slit bandwidths were set at either 5 nm or 10 nm depending on the SMP concentration in the sludge samples. The emission spectra were set between 200-500 nm while the excitation spectra were between 220-380 nm. The fluorescence regional integration was then performed to quantify the fluorescence intensity corresponding to each component by calculating the area under the peak in their respective excitation/emission wavelength regions [186, 187]. The fluorescence scan was performed using the Cary Eclipse Scan software (Cary Eclipse Scan Version:1.2 (147)), while FRI measurement was performed using the Cary Eclipse Maths Calculator software (Cary Eclipse Scan Version:1.2 (147)). The SMP, LB EPS and TB EPS fractions were viewed at different voltages. Thus, only intra-fraction comparisons were feasible, and the results were analysed accordingly.

CHAPTER 3

Synchronous fluorescence analysis of the EPS samples was performed between 200 to 400 nm with a constant difference ($\Delta\lambda$) between excitation and emission wavelength of 60 nm [188, 189].

CHAPTER 4

CHAPTER 4

Evidence for the resistance of activated sludge flocs to deflocculative DO stress

4.1 Introduction

At a wastewater treatment plant (WWTP), formation and maintenance of large dense flocs (flocculation) are of utmost importance in order to improve liquid-solids separation. The stability, integrity and strength of the flocs are dependent on multiple physical, chemical and biological factors. Adversities induced by these factors can induce the disruption and break-up of biological flocs, i.e. deflocculation. This deflocculation can be in the form of floc erosion, which consists of the breaking off of floc components in the outer layers of the floc body, or floc fragmentation, which results in the break-up of larger parts of the floc body [14, 15]. Floc erosion leads to an increase in the number of very small particles in the bulk solution while floc size and floc size distribution is not greatly affected. Meanwhile, floc fragmentation results in a more significant floc size reduction and a more noticeable change in the floc size distribution [148]. In both cases, liquid-solids separation is adversely affected leading to increased supernatant turbidity and suspended solids which reduces effluent quality and creates more issues in downstream treatment processes [14, 15, 190].

Reported studies have shown that deflocculation can be induced in a very short time frame (within 2 to 4 hours) upon exposure of sludge flocs to low DO (≤ 0.5 mg/L) or anaerobic conditions. The sudden exposure to such DO conditions affected the viability and activities of floccular microbial communities, which in turn influenced the stability and integrity of sludge flocs. The aerobic and anaerobic microbial activities have been reported to affect the quantity, chemical composition and

CHAPTER 4

intramolecular interactions of the extracellular polymeric substances (EPS) in the sludge [191, 192]. EPS is the “glue” synthesized by microbes in the sludge that help in formation and stabilization of the floc structure [127, 193]. The quantity and quality of EPS synthesized strongly influence the bio-flocculation [64], floc type [157, 170], floc strength [157, 170] and resistance to stress and toxins [127]. EPS production has been reported to be increased under aerobic and high DO conditions [64, 194]. This correlated with the improvements in flocculation potential and rate and resulted in reduced supernatant turbidity and increased floc settleability [64, 194]. Increased amounts of bound EPS under high DO conditions (>4 mgDO/L) are reported to be responsible for the improvements in floc stability and resistance to stresses through their interactions with floc components, cations and environmental toxins [17, 18, 127, 193, 194]. The bound EPS proteins especially aid in floc stabilization through their hydrophobic [37, 193] and ionic interactions [194]. The structural proteins synthesized under aerobic conditions also aid in the formation and maintenance of floc structure [38]. Conversely, deflocculation observed under anaerobic conditions was linked to reduced microbial activity and EPS production [12, 15] along with the synthesis of more hydrolytic proteins [38], which would disrupt the floc stabilizing interactions. Other floc destabilizing anaerobic microbial activities include the reduction of ferric iron to ferrous iron as a result of anaerobic respiration, which will reduce the number of interactions in the sludge flocs [12, 15]. It has also been shown subsequent aeration of the deflocculated sludge induced re-flocculation. This, it was speculated, was due to increased microbial activity (and EPS production) under the aerobic conditions [12, 15].

CHAPTER 4

This experimental study was undertaken following the authors' earlier observations deflocculation had not occurred under low DO (<0.5 mg/L) conditions. This was contrary to expectations and the reported literature [14, 15]. Repeated experiments had then consistently demonstrated sludge deflocculation under low dissolved oxygen (DO) conditions (<0.5 mg/L) is not a universal phenomenon. This finding is potentially important in wastewater treatment practise and also serves to alert on possible need to review current assumptions on flocculation. The experiments in this study were designed to provide opportunity to study flocculation and deflocculation under controlled conditions and with minimal sample disruption caused by processes not considered a variable in this study. In addition to supernatant turbidity monitoring, changes in the bound and soluble EPS and their components were determined to allow study of their changes with respect to various DO conditions and deflocculation. This was expected to provide a more holistic and accurate assessment of the link between DO levels and the floc stability.

4.2 Materials and methods

4.2.1 Sludge samples

The experiments were performed with sludge samples collected from a laboratory scale 8-litre sequencing batch reactor (SBR) fed with synthetic wastewater. The synthetic wastewater was composed of glucose (400 mg/L COD), ammonium chloride (40 mg/L $\text{NH}_4^+\text{-N}$) and potassium di-hydrogen phosphate (15 mg/L $\text{PO}_4^{3-}\text{-P}$) respectively. Sodium bicarbonate (640 mg/L) was used as buffer. Calcium chloride (9.25 mg/L Ca^{2+}), magnesium sulphate (2 mg/L Mg^{2+}), ferrous sulphate (0.04 mg/L Fe^{2+}), nickel chloride hexahydrate (0.0074 mg/L Ni^{2+}), zinc chloride (0.058 mg/L

CHAPTER 4

Zn^{2+}), cobalt chloride hexahydrate (0.015 mg/L Co^{2+}), copper sulphate pentahydrate (0.0076 mg/L Cu^{2+}), ammonium molybdate (0.0033 mg/L $\text{Mo}_7\text{O}_{24}^{6-}$), potassium iodide (0.023 mg/L I), manganese sulphate dihydrate (0.018 mg/L Mn^{2+}) and boric acid (0.015 mg/L) were added to provide the trace elements necessary for effective floc formation. The SBR was operated with a programmable logic controller (PLC) for process control. The SBR cycle had Anoxic Fill, Anoxic React, Aerobic React, Sludge Wasting, Settle and Decant. The anoxic period was 52 minutes with mixing, aerobic period was 212 minutes, and total cycle time was 6 hours. The exchange ratio in each cycle was 50% and hydraulic retention time (HRT) was 12 hours. Mixed liquor temperature in the SBR was maintained at the room temperature of 25°C with a heating tape. DO concentration during the aerobic period was controlled at 1 to 2 mg/L and pH was maintained at between 7 and 8 by dosing hydrochloric acid (HCl) or sodium hydroxide (NaOH). The solids retention time (SRT) in the SBR was maintained at 15 days by discharging a calibrated volume of sludge during sludge wasting. After 3 SRTs (45 days), sludge was collected for the batch tests.

The WWTP sludge samples utilized in the batch tests were collected from the aerobic tanks at the WWTPs. The activated sludge process in both WWTPs were operated in the Modified Ludzack-Ettinger (MLE) configuration for nutrients removal. The WWTP sludge samples were brought from the treatment plants to the laboratory within one hour of collection. In the laboratory, the collected sludge was settled, and decanted to reduce volume by 50%. At the start of experiment, synthetic wastewater was fed to the settled sludge to simulate anoxic feeding at the start of the MLE process. The final TSS concentration was adjusted to 1.5 g/L.

CHAPTER 4

4.2.2 Experimental protocol

Batch tests were conducted to study the effect of low DO (AN), aerobic (OX) and alternating low DO/aerobic (AN-OX) conditions on flocculation and deflocculation with sludge from the three sources, i.e. the laboratory-scale SBR and the two WWTPs in Singapore. Low DO conditions in this study referred to DO concentration at 0.5 mg/L or lower, while aerobic conditions referred to DO levels higher than 0.5 mg/L. Samples were ultrasonicated, in duplicates, to produce deflocculated positive controls. The batch test conditions were tested in duplicates. Conditioning and tests were performed at laboratory ambient temperature of 25 °C, unless otherwise specified.

For each round of testing and for each sludge, 10 samples of 80 mL each with total suspended solids (TSS) of 2.1 g/L, were placed in 100 mL bottles. The SBR sludge was collected after the feeding phase, while the WWTP sludges were placed directly into the bottles just before the experiment was started. Two of the samples were immediately analysed as “zero-hour controls”. Other samples were purged with nitrogen to clear the headspaces of air and closed with air-tight caps. Two of them were ultrasonicated (homogenized as per the procedure mentioned below), while the remaining six were shaken at 190 rpm for one hour. The samples were thus subjected to low DO conditions.

Homogenization (H) of two of the samples was performed with ultrasonication - pulsing at 37 KHz and 80% power (Power Effective – 330 W, Peak performance max. – 1320 W) for 30 minutes in an ultrasonication water bath (Elmasonic P Ultrasonic Cleaner-Model 180), which was maintained at 50°C.

CHAPTER 4

The samples were subjected to one hour of low DO conditioning to reduce the DO levels in the samples to below 0.5 mg/L and to standardize conditions in all the sludge samples before exposing them to various DO conditions. The “zero-hour control” samples were subjected to DO, pH, ammonium, turbidity and EPS measurements and served as comparison against the other sludge samples. The samples were analysed after the first hour of low DO conditioning and subsequently after every two hours.

DO, pH and ammonium readings were measured using electrochemical probes inserted directly into the bottles. After the measurements were made, the samples were mixed for 5 seconds and then left to stand to allow settling (low DO conditioned samples were additionally purged with nitrogen in their headspace and capped again). After 10 minutes of settling, 10 mL of the supernatant was collected with an automatic pipette for turbidity measurements. After measurement, the supernatant samples were returned to the respective sample bottles. The low DO sample bottle headspaces were purged with nitrogen after every opening for sampling.

After each sampling, the samples were returned to either low DO or aerobic shaking at 190 rpm and at room temperature. The homogenized samples were subjected to aerobic shaking (H-OX) to assess the reflocculation phenomena.

4.2.3 Analytical Methods

The TSS, VSS, DO, pH and ammonium content were measured as specified in Chapter 3. Supernatant turbidity was measured with a turbidimeter (Hach, 2100N) in terms of Nephelometric Turbidity Unit (NTU).

CHAPTER 4

Supernatant samples were filtered with a 0.45 μm filter and the filtrate was considered to hold the soluble microbial products (SMP) fraction. The bound EPS fractions were extracted with a modified version of the mild heat extraction method as specified in Chapter 3 [157, 183]. The colorimetric and FEEM analysis of the SMP and bound EPS were performed in accordance with the methods specified in Chapter 3.

4.3 Results

4.3.1 SBR sludge

Fig. 4.1 shows the SBR sludge conditions and performance in terms of DO, ammonium, pH, and supernatant turbidity during the limited DO, alternating, and aerobic conditions. DO concentration in AN samples was maintained below 0.5 mg/L, while for the OX samples the DO concentration had increased to more than 5 mg/L. This indicated that oxygen was available in the OX samples and this oxygen will be utilized by the microbes for aerobic respiration, which will promote effective microbial growth and metabolism along with nitrification. The ammonium concentration was low (1.8 mg/L) when the sludge was collected from the SBR after the 30 minutes feeding period. In the opened bottles of OX samples, pH had increased to around 8, and this was in part due to carbon dioxide stripping. After changing from aerobic to DO limited conditions, pH was reduced to about 7, and this was due to the accumulation of acidic metabolic by-products (e.g., carbon dioxide, organic acids).

CHAPTER 4

Figure 4.1: Changes in (a) DO, (b) ammonium, (c) pH and (d) turbidity of SBR sludge samples (AN, AN-OX, OX and H-OX) during the batch test.

The supernatant turbidity levels were comparable among all the samples throughout the batch test. This was the case regardless of the DO levels in the system and the duration of exposure to the low DO levels (i.e. more than 11 hours). No noticeable change in supernatant turbidity had occurred even after another 18 hours of low DO conditions and this was all in the absence of fresh feed supplementation at any point in

CHAPTER 4

the batch test (i.e. expected to lead to starvation). These results are contradictory to the available literature [15], where low DO levels have been reported to result in deflocculation (increased turbidity) while higher DO (5 mg/L) levels promoted better flocculation with lower supernatant turbidity and suspended solid content [129]. The turbidity results in this study are indicative of a lack of deflocculation in the SBR sludge samples. At the end of the experiment, the samples were analysed for particle size distribution and composition of the different EPS fractions (Fig. 4.2a and b). The flocs of the different DO conditioned samples were also of similar size and the EPS content, bound and unbound EPS, were similar in composition (Fig. 4.2a and b). These results further corroborate the lack of deflocculation in the different DO conditioned samples.

The same analytical protocol was applied to the samples mechanically stressed by ultrasonication. The deviation among the ultrasonicated duplicates is likely due to the uneven distribution of dispersing energy in the ultrasonication bath. However, since the purpose of this treatment was to show the sludge's susceptibility to deflocculation, the variation among the samples is of less importance to the overall study objective. Ultrasonication shear had resulted in significantly higher supernatant turbidity (Fig. 4.1d). The ammonium level in the supernatant had increased to and then remained at about 5 mg/L till the end of the experiment indicating suppression in microbial activities. Despite the increased supernatant turbidity in the ultrasonicated samples, the flocs were not reduced in size (Fig. 4.2a). This indicated the occurrence of floc erosion and not floc fragmentation in the ultrasonicated samples. The reduction in supernatant turbidity of the ultrasonicated samples over the duration of the batch test indicated the

CHAPTER 4

occurrence of re-flocculation when subjected to highly aerobic conditions (<5 mg/L). This re-flocculation under high DO conditions was in agreement with previous reported literature [14, 15].

Analysis of the SMP and EPS fractions showed that 80% of the extractable EPS comprised the TB fraction and mainly containing proteins and humic acid. Proteins are considered to be mainly responsible for promotion and maintenance of flocculation in activated sludge flocs [38, 193] and therefore, their high content in the extracted EPS was likely the reason for strong and stress-resistant flocs. In the case of deflocculation, the interactions mediated by EPS are expected to gradually weaken leading to its break-up from the floc body. In such situations, increased levels of SMP with reducing TB EPS could then be expected. This was shown to be so with the positive control samples but not in the AN samples (Fig. 4.2b).

CHAPTER 4

Figure 4.2: Measurements of the SBR sludge samples at the end of the batch test: (a) particle-size distribution; (b) chemical composition of SMP, LB EPS, and TB EPS fractions according to colorimetry; and (c) chemical composition of SMP, LB EPS, and TB EPS fractions according to FEEM measurements. The FEEM measurements were performed at 10 nm excitation/emission slits for SMP and LB EPS fractions. For the TB EPS fractions, FEEM measurements were performed at 5 nm excitation/emission slits.

On top of the conventional colorimetric analysis, FEEM was employed to analyse the various EPS fractions. The FEEM technique allowed assessment of the EPS based on the unique excitation/emission signals of only its fluorescent components and so differentiating it from the colorimetric method. The FEEM analysis showed high similarity of the SMP and extractable bound EPS fraction distribution between the AN-OX and OX samples (Fig. 4.2c). In the AN samples, the total fluorescence

CHAPTER 4

intensity for SMP fraction was observed to be higher compared to the AN-OX and OX samples, but lower in the LB EPS fraction. Trp/Tyr containing proteins and amino acids were the dominant fluorophores across all the analysed samples. The lower contribution of Trp/Tyr containing amino acids in the SMP fraction of OX sample was likely due to the utilization of amino acids as nitrogen source for more active microbial metabolism [195]. The differences in the contribution of fluorescent fulvic acid between AN and OX samples was likely due to higher microbial decomposition of fulvic acid under aerobic conditions [196]. Analogous to the colorimetric results, the FEEM data showed trends of EPS dissolution in H-OX samples with lower total integral fluorescence for TB EPS fraction in comparison to the other samples. The total fluorescence intensity of H-OX SMP fractions was more than double that of the other samples. Each fraction of the extractable bound EPS, including the TB EPS fraction, was rich in fluorescent proteins indicating there was still a protein-rich pool of non-extractable EPS within flocs of the sludge samples.

4.3.2 WWTP 1 sludge

A similar experiment was performed on sludge collected from a local municipal WWTP in Singapore (WWTP 1). As seen from Fig. 4.3b, this sludge was also active in ammonium removal. Ammonium readings for the “zero-hour control” samples were reduced from 21.6 mg/L to 10.8 mg/L despite a much shorter feeding time compared to the SBR sludge. The DO levels of the AN samples were maintained well below 0.5 mg/L throughout the test while oxygen was available for the OX samples. Ammonium removal was observed in the AN samples despite the low DO concentration. After the first two hours of aerobic treatment, the DO level was also relatively low for the AN-

CHAPTER 4

OX and OX samples, which was indicative of high rates of oxygen consumption and hence microbial activity. pH of all samples was in the range of 7 to 8 throughout the experiment.

Figure 4.3: Changes in (a) DO, (b) ammonium, (c) pH and (d) turbidity of WWTP 1 sludge samples (AN, AN-OX, OX and H-OX) during the batch test.

No significant changes in turbidity or particle size distribution were observed for the non-ultrasonicated samples (similar to the experiment with SBR sludge). The

CHAPTER 4

supernatant turbidity in the ultrasonicated (H-OX) samples increased to more than 300 NTU immediately after ultrasonication. However, the supernatant turbidity was substantially reduced during the subsequent aerobic conditioning of the H-OX samples indicating effective re-flocculation of the sludge samples. The ammonium concentration of the H-OX samples had gradually decreased over the duration of the batch test, which together with the suppressed DO concentration indicated high microbial activity in the ultrasonicated samples. The viable microbes and their activities could be expected to have induced the significant re-flocculation in the WWTP 1 sludge samples. pH of the H-OX samples went to around 8 (the same as for OX samples) after the depletion of ammonium. The floc size distribution profile revealed the flocs in the H-OX samples were smaller than their counterparts in the non-ultrasonicated (AN, AN-OX and OX) samples further corroborating the deflocculation in the H-OX samples (Fig. 4.4a). However, for this sludge, a noticeable decrease in floc size was also observed for AN samples.

CHAPTER 4

Figure 4.4: Measurements of the WWTP 1 sludge samples at the end of the batch test: (a) particle-size distribution; (b) chemical composition of SMP, LB EPS, and TB EPS fractions according to colorimetry; and (c) chemical composition of SMP, LB EPS, and TB EPS fractions according to FEEM measurements. The FEEM measurements were performed at 10 nm excitation/emission slits for SMP and LB EPS fractions. For the TB EPS fractions, FEEM measurements were performed at 5 nm excitation/emission slits.

FEEM analysis revealed increased levels of fluorescent SMP components in the H-OX and AN samples which was consistent with the floc size distribution findings discussed above (Fig. 4.4c). In the H-OX samples, the increase was mainly brought about by Trp/Tyr containing proteins while in the AN samples, the fluorescence increase was more proportionally distributed across all the components. Moreover, the fluorescent intensities of TB EPS fractions were smaller in the H-OX samples,

CHAPTER 4

suggesting dissolution and break-up of bound EPS. In the AN samples, the composition of the TB EPS remained similar to the other samples. This suggested the higher levels of Trp/Tyr containing amino acids and fulvic acids might have been a result of microbial activity and not from the solubilization of bound EPS. The colorimetric results only confirmed increased levels of SMP in the H-OX samples (Fig. 4.4b). Therefore, deflocculation of the AN samples should be considered with caution. In the OX samples, the lower total fluorescent intensity, along with the lower fraction of amino acids [195] and the higher level of polysaccharides (SMP-like) was likely due to higher aerobic metabolism in these samples. In general, the composition of SMP and EPS of the WWTP 1 sludge was found to be very similar to the SBR sludge with proteins and amino acids being the major fluorophores of every fraction of EPS. The protein-rich microbial flocs resisted significant deflocculative tendencies ensuring low supernatant turbidity under low DO conditions. However, the observation of increased fluorescent SMP content may be indicative of the beginning of deflocculation process in the AN samples, but this was the case only after 24 hours of low DO conditioning. Contrary to the SBR sludge sample, the WWTP 1 H-OX samples exhibited strong microbial activity and efficient reduction of supernatant turbidity over the duration of the batch test. However, results of bound EPS analysis and the floc size distribution indicated the re-flocculation was not complete within the experimental time frame.

4.3.3 WWTP 2 sludge

Ammonium removal rate for sludge from another municipal WWTP in Singapore (WWTP 2) was lower (Fig. 4.5b). The lower ammonium removal rate of the WWTP 2

CHAPTER 4

sludge samples were likely due to differences in microbial population resulting from the different operation strategies. As ammonium removal was prioritised in WWTP 1 and the laboratory SBR, the microbial population targeting nutrients removal was expected to be higher there than in WWTP 2. DO was maintained at high concentration in the OX samples throughout the experiment and the pH trend during the first 7 hours was similar to the batch experiments of the previous two sludges (Fig. 4.5a &c). After 7 hours of aerobic conditions however, the pH of aerobic samples started to decrease (Fig. 4.5c). This phenomenon was not observed in the ultrasonicated samples, which were also exposed to constant aeration similar to the aerobic samples. No convincing explanation could be found for the observed phenomenon. The pH drop was not expected to adversely affect sludge flocculation (since pH was greater than 5 even after the pH drop) in the OX samples. The supernatant turbidity in the non-ultrasonicated samples were low throughout the batch test and were comparable among AN, AN-OX and OX samples (Fig. 4.5d), which indicated a lack of deflocculation due to low DO conditioning.

CHAPTER 4

Figure 4.5: Changes in (a) DO, (b) ammonium, (c) pH and (d) turbidity of WWTP 2 sludge samples (AN, AN-OX, OX and H-OX) during the batch test.

As with earlier tests, ultrasonication led to an increase in supernatant turbidity. One of the H-OX samples exhibited significantly smaller floc size than the other, indicating the possibility of significant floc fragmentation in that sample alone (Fig. 4.6a). When analysed along with turbidity data, it was clear that ultrasonication had been more severe in one of the H-OX samples and significant floc size reduction had occurred in this sample. In the other H-OX sample, floc erosion was expected to have occurred contributing to the increased supernatant turbidity. The floc size distribution profile of the non-ultrasonicated samples indicated comparable floc sizes. This corroborated the lack of significant deflocculation in low DO conditioned samples.

CHAPTER 4

Figure 4.6: Measurements of the WWTP 2 sludge samples at the end of the batch test: (a) particle-size distribution; (b) chemical composition of SMP, LB EPS, and TB EPS fractions according to colorimetry; and (c) chemical composition of SMP, LB EPS, and TB EPS fractions according to FEEM measurements. The FEEM measurements were performed at 10 nm excitation/emission slits for SMP and LB EPS fractions. For

CHAPTER 4

the TB EPS fractions, FEEM measurements were performed at 5 nm excitation/emission slits.

With the WWTP 2 sludge, FEEM analysis showed the SMP composition of AN-OX samples were more similar to AN samples than the OX samples. This suggested possibility of higher sensitivity of this sludge to low DO levels (Fig. 4.6c). The OX samples again contained comparatively lower fractions of Trp/Tyr containing amino acids and fulvic acids, which was expected to be due to their utilization by microbes under aerobic conditions [195, 196]. Aerobic microbial activity was also expected to be the reason for lower total fluorescent intensity in the OX samples. The higher fluorescent intensity in the LB EPS fraction of AN-OX 2 sample was expected to be due to the extraction of excess LB EPS during the EPS extraction and not due to their batch test conditioning. The overall composition of the SMP and bound EPS for WWTP 2 sludge samples were very similar to the previous sludge samples with Trp/Tyr containing proteins and amino acids being the major fluorophores and the most abundant (more than 80%) EPS fraction was the TB EPS (Fig. 4.6b). Again, for the H-OX samples, substantial increase in the SMP along with a decrease in TB EPS content, which together with reduced average floc size (Fig. 4.6a) suggested deflocculation through solubilization of bound EPS.

4.4 Discussions

Deflocculation can be termed as the break-up of components from the floc body leading to the increase in supernatant turbidity, suspended solids and soluble microbial products along with a simultaneous decrease in floc size [15, 46]. This floc size decrease could be less pronounced as in the case of erosion of the components from

CHAPTER 4

outer layers of the floc or be more significant when fragmentation of the floc body occurs. These indicators must be assessed in order to more accurately determine the occurrence of sludge deflocculation. The non-ultrasonicated samples did not exhibit the typical symptoms of deflocculation regardless of the DO levels in the system while the physically disrupted sludge samples were deflocculated to different extents. The reasoning behind these results will be discussed as follows.

Deflocculation was observed when the sludge samples were subjected to ultrasonication stress at high temperature (50 °C). This was expected to be due to adverse effects of the high temperature and ultrasonication stress on the bound EPS, especially the TB EPS, and their interactions. The higher levels of SMP and, at the same time, reduced content of TB EPS in these physically disrupted samples corroborate the break-up and solubilization of the TB EPS as being a major mechanism of deflocculation. Morgan-Sagastume and Allen [46] reported a similar EPS solubilization-based deflocculation when they subjected the sludge samples to 45 °C. Thus, sludge deflocculation in this study occurred only when a significant stress was induced on the floc EPS and their interactions.

Analysis of the extractable bound EPS composition of all sludge samples in this study showed high content of proteins and they were most abundant in the tightly bound fraction, which made up the majority of the bound EPS content. The literature has reported EPS proteins are responsible for influencing the stability and the integrity of the floc [38] and the tightly-bound EPS fraction is usually dominated by proteins [175, 193]. The tightly bound EPS, especially its protein content, has been reported to be responsible for promoting sludge aggregation through hydrophobic interactions [193].

CHAPTER 4

Basuvaraj, Fein [197] reported the presence of higher total TB EPS and TB EPS protein content in the granular flocs observed in their study while also observing an inverse relationship between the SVI and TB EPS/LB EPS ratio. The sludge samples in this study which had well developed flocs also had abundant TB EPS rich in proteins. These strong and stable flocs were able to resist adverse DO conditions. This suggests deflocculation cannot be as easily defined as previously thought and that well-developed sludge flocs might be able to resist short-term deflocculative stresses (such as low DO conditions). Further work on this would be essential as development and maintenance of well-developed sludge flocs is likely more effective compared to subsequent mitigation of deflocculation.

It is necessary to note however, that there are various other factors that could contribute to the different outcomes of low DO conditions on sludge flocculation as observed in this study and reports in the literature. All samples in this study were fed before the experiments unlike in the previous studies [14, 15]. This was to prevent starvation effects while also promoting microbial growth and metabolism. The increased growth and metabolism would promote higher EPS production which in turn will aid in improving floc stability and integrity [11, 12, 14, 15]. The microbes cultured in the SBR and even in many WWTPs are not just strict aerobes and anaerobes, but facultative bacteria that can utilize either oxygen or other electron acceptors for their respiration depending on the prevailing conditions. These bacteria can metabolize and even produce EPS under low DO and anaerobic conditions [198]. Wilén, Keiding [14] reported the mitigation of deflocculation under anaerobic conditions upon the addition of nitrate, which was speculated to be due the utilization

CHAPTER 4

of nitrate by some aerobes as an alternate electron acceptor for their metabolism. The bacteria capable of utilizing nitrate in such a manner are generally not strict aerobes, but are more often facultative like *Shewanella* sp.. Thus, considering the reports in literature, the facultative bacterial activities were speculated to have counteracted the reduced activities of strict aerobes under these conditions. This could explain the lack of deflocculation under low DO conditions in this study.

Another possible difference might be due to the ionic content of the synthetic wastewaters used (Ca^{2+} , Mg^{2+} and Fe^{2+}) and the sludge conditioning. Sludge grown under different electrolytic conditions can be expected to result in flocs of varying physical properties due to the interactions of respective ions with the EPS [15, 18, 74, 80]. Multivalent (di- and tri-valent) cation additions have been reported to promote better floc interactions [17, 18, 194] and protect the EPS in the floc matrix [134] when compared with the monovalent cations. These interactions with multivalent cations resulted in stronger and more stress resistant flocs. The tri-valent cations formed stronger interactions with the EPS than the divalent cations. The redox activities of iron, which will lead to changes in its valency, can influence the flocculation process greatly through its varying strength of interactions with the EPS and also through the generation of free radicals as a by-product [123]. Wilén, Keiding [14] had reported the reduction of Fe(III) to Fe(II) under anaerobic conditions, especially in the presence of facultative *Shewanella* sp.. This had decreased the EPS bridging capacity and made the flocs more susceptible to other stresses bringing about deflocculation. The SBR sludge in this study was conditioned with much less iron ($<1 \mu\text{mol/gVSS}$) than in the other studies (sludge containing total Fe concentrations of 1 mmol/gVS or 2.7

CHAPTER 4

mmol/gVS) [11]. Therefore, the effects of iron reduction on the stability of the sludge flocs can be expected to be much less influential in this study and this could have influenced the lack of deflocculation under low DO conditions. Unfortunately, the ionic compositions of the activated sludge samples drawn from the WWTPs were not assessed for comparison with the SBR sludge. The ionic content (divalent calcium and magnesium) of the synthetic feed added to the sludge samples could have also influenced the floc structures. However, it was not expected to have influenced as much as their initial conditioning in the WWTPs and SBR reactor.

Finally, the experimental protocol itself could be a source of bias in flocculation studies. In the reported literature [14, 15] the different levels of DO were obtained by purging the samples with nitrogen or air, while solids separation was achieved through centrifugation. Shear stress is known to have an adverse impact on floc size and integrity [12, 14]. The gas bubbles formed during the purging will induce additional shear, and this can influence sludge samples in a varying fashion depending on the initial floc structures. Even though gas purging was speculated to not induce adverse shear effects on the sludge flocs in other studies, it led to greatly increased levels of standard deviation between replicates when similar protocols were employed in this study in its early stages (data not shown). This deviation was especially observable with the supernatant turbidity levels, which was expected to be due to erosion of the flocs by gas bubbles. This led to the protocol used in this study, which avoided the impact of gas purging induced shear on the flocs without compromising on the main objective of assessing the effects of varying oxygen levels on the sludge flocculation. Additionally, these changes also improved consistency of results. Based on the results

CHAPTER 4

obtained, the choice of experimental and analytical methods must be carefully considered as this can also have an impact on the flocculation results.

4.5 Conclusions

Sludge sampled from a laboratory scale SBR and two full scale municipal WWTPs were studied to assess susceptibility to deflocculation when exposed to low DO conditions. It was observed that regardless of the sludge type neither prolonged low DO (and even anaerobic in later stages) nor starvation conditions led to deterioration of floc structure or supernatant quality. The severe deflocculation effects under anaerobic conditions reported in the literature were not observed in this study. Analysis of the SMP and bound EPS fractions revealed that all the analysed sludge samples showed high content of proteins and the tightly bound fraction was the most abundant fraction regardless of the conditions the samples were exposed to. The TB EPS content of the sludges suggested well-developed flocs with strong and stable interactions which could be expected to better resist the deflocculative stresses brought about by low DO conditions. The turbidity, floc size distributions and the EPS fractions (SMP and bound EPS) were noted to be good indicators of deflocculation. In this study, only through physical disruption, using ultrasonication, was deflocculation achieved. This was observed to be partially reversible as subsequent exposure to high DO led to a partial reflocculation event within the experiment's timeframe.

The contrast between the results presented in this work and the reported literature was likely due to differences arising from varying in situ environmental conditions, microbial communities (aerobic, facultative or anaerobic), floc compactness, ionic content and the experimental protocol used (with regards to the shear stresses applied).

CHAPTER 5

CHAPTER 5

Sodium azide inhibition of microbial activities and impact on sludge floc destabilization

5.1 Introduction

Effective biological flocculation encompasses both floc formation and its maintenance, and these phenomena are influenced by microbes in the sludge and their by-products, i.e. extracellular polymeric substances [11, 192]. Adverse impacts on these microbial communities, the EPS and their interactions are thus reported to be a major cause for disruption of floc stability and sludge deflocculation. Considering the importance of microbial activities, the dissolved oxygen levels in the treatment processes have a major impact on the flocculation process. The increased amounts of EPS (especially proteins) propagated under conditions of high DO have been reported to contribute to the build-up, stabilization and maintenance of the floc body through their several floc stabilizing interactions [38, 64, 193, 197]. This also provides the floc with the strength to resist deflocculative stresses [127].

Anaerobic and low DO conditions ($\text{DO} < 0.5 \text{ mg/L}$) on the other hand have been reported to bring about deflocculation due to factors like reduced aerobic microbial activity [14], stimulated anaerobic microbial activity [11], reduced EPS production [15] and increased hydrolytic protein production [38]. The reported deflocculation occurs as early as 1 to 2 hours after exposure to the low DO or anaerobic conditions, which indicates a rapid impact of the DO transients on sludge floc stability [11, 14, 15]. It has to be noted the deflocculation in these reported studies were concluded mostly based on increased supernatant turbidity and soluble microbial products (SMPs), while the changes in floc size were not assessed. This is important because

CHAPTER 5

changes in turbidity and SMP levels could be brought about by changes to the planktonic bacteria alone, while the flocs themselves were unaffected. The low DO deflocculation results must thus be considered with some caution and more studies required to confirm the phenomena.

Recent studies have suggested need for reconsideration of the assumptions made on biological flocculation [199], especially under low DO conditions (≤ 0.5 mg/L). In the authors' earlier study, observed sustained performance of floc was attributed to compensation of reduced aerobic microbial activity by the facultative bacteria in the absence of deflocculation [199]. To further elucidate the latter, influence of overall microbial activities on the flocculation process can be observed by inhibiting their activities and evaluating the subsequent effects. Utilizing this strategy under low DO conditions can indicate the impact facultative bacteria have on the flocculation process and, this in turn, can help explain the absence of low DO deflocculation reported in the previous study [199]. A similar strategy was utilized by Wilén, Lund Nielsen [11] in their study inhibiting sludge microbial communities using sodium azide and subsequently reported the occurrence of deflocculation in terms of increased supernatant turbidity. However, it has to be noted Wilén, Lund Nielsen [11] did not monitor the floc size changes nor the changes in EPS content while performing their experiments, which made their speculation of deflocculation inconclusive. Sodium azide is a highly reactive compound that is reported to even induce cell lysis [200]. The increased turbidity could thus be contributed by the lysis of planktonic bacteria while the floc itself might have remained unaffected by sodium azide spiking.

CHAPTER 5

Deflocculation can only be properly confirmed by monitoring the floc size distribution in the azide spiked samples, which was not the case with their work [11].

Thus, in this study sodium azide was used to inhibit the microbes under both high and low DO conditions, and the changes in microbial activities, floc size, supernatant turbidity, unbound and bound EPS content were monitored to properly assess its impact on the flocs. The sodium azide was also evaluated for its interactive capability with bound EPS content, which would indicate another mechanism through which it could influence the floc integrity. These experiments would allow for a more accurate evaluation of the impact of sodium azide influenced microbial inhibition on the floc stability and integrity.

5.2 Materials and methods

5.2.1 Sludge samples

The experiments were performed with sludge samples collected from a laboratory scale 8-litre sequencing batch reactor (SBR) fed with synthetic wastewater, as specified in section 4.2.1 of Chapter 4.

5.2.2 Experimental setups

Batch test was conducted to assess the effect of sodium azide on biological floc stability under high DO conditions ($\text{DO} \geq 1$ mg/L). The sludge samples were dosed with various azide concentrations for this purpose: 0, 120, 360 and 720 mg NaN_3/g VSS and then subjected to aerobic conditions ($\text{DO} \geq 1$ mg/L). The experimental conditions were tested in duplicates. 8 samples of SBR sludge of 100 mL, each with volatile suspended solids of 2 g/L, were placed in 100 mL bottles. The sludge was

CHAPTER 5

collected at the end of the aerobic react phase, settled for 30 minutes after which 50 mL of the supernatant was discarded leaving behind 50 mL of settled sludge. The discarded supernatant was replaced with synthetic wastewater feed and sodium azide. The latter was drawn from a 10 g/L stock solution to bring about the required azide concentrations. All the samples were subjected to “zero-hour” soluble COD, DO, pH, ammonium, potassium and turbidity measurements. Subsequently, the samples were shaken at 190 rpm in open bottles to allow for diffusion of oxygen from air into the sludge samples to bring about the required aerobic conditions. The sludge conditioning and analysis were performed at 25 °C, unless otherwise specified.

For the batch test assessing the effect of sodium azide on sludge flocs under low DO conditions, 8 samples of SBR sludge of 100 mL were collected at end of the aerobic react phase and placed in 100 mL bottles. The volatile suspended solids content of each of the sludge samples were 2.8 g/L. Two of the samples were analysed as zero-hour control duplicates. The remaining samples were subjected to the experimental test conditions (OX, AN and AZ-AN) in duplicates. The SBR sludge was settled for 30 minutes after which 50 mL of the supernatant was discarded. The discarded supernatant was replaced with the synthetic wastewater feed and sodium azide at a concentration of 120 mg/gVSS (or with DI water in the case of the control samples). The low DO control (AN) and the azide added samples (AZ-AN) were purged with nitrogen in their headspaces and closed with air-tight caps to bring about low DO conditions. The high DO control (OX) samples were shaken in open bottles to allow for diffusion of oxygen from air into these samples to maintain aerobic conditions. Subsequently, all the samples were shaken at 190 rpm with pH, ammonium, potassium

CHAPTER 5

and turbidity monitoring every two hours. The sludge conditioning and analysis were performed at 25 °C, unless otherwise specified.

Experimental measurements were made every two hours. DO, pH, potassium and ammonium readings were measured using electrochemical probes inserted directly into the bottles. 2 mL of sludge samples were collected and filtered for dissolved organic carbon, nitrate and nitrite measurements. All the samples were then mixed manually and left to stand undisturbed for 10 minutes, after which 1.5 mL of the supernatant was collected for turbidity measurements. After the turbidity measurements, the supernatant samples were returned to the respective sample bottles prior to open bottle shaking at 190 rpm. The AN and AZ-AN samples were purged with nitrogen in their headspaces during each measurement to maintain the low DO conditions and were closed with air-tight caps prior to shaking.

The sodium azide was reacted with extracted (loosely bound and tightly bound) EPS to assess possible interactions between them. The interaction tests were performed in duplicates. The control samples consisted of 500 µL of extracted EPS made up to 5 mL with DI water in 50 mL Eppendorf tubes. The sodium azide spiked samples consisted of 500 µL of extracted EPS made up to 5 mL with DI water and various amounts of sodium azide. All the sample tubes were capped and shaken at 250 rpm at room temperature for 3 hours. Subsequently, the samples were analysed using a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, USA). FEEM analysis was utilized to assess the occurrence of interaction, based on the quenching of EPS fluorescence.

CHAPTER 5

5.2.3 Analytical Methods

TSS, VSS, DOC, DO, pH, ammonium concentration, potassium concentration, nitrate concentration, nitrite concentration, turbidity and floc sizes were measured as specified in Chapter 3. The supernatant turbidity measurements were in terms of absorbance at 650 nm measured with a UV spectrophotometer (UV-1800, Shimadzu, Japan). The mean floc size and floc size distribution profiles were utilized to express the changes in the floc sizes among samples. The SMP and bound EPS were extracted by the mild heat extraction protocol as specified in Chapter 3. The colorimetric measurements and FEEM analysis of the EPS fractions were performed in accordance with the methods specified in Chapter 3. UV-absorption spectra of the EPS and EPS-azide complexes was recorded between 200 to 800 nm using a double beam UV-Vis Spectrophotometer [189].

5.3 Results

5.3.1 Influence of sodium azide on flocculation under high DO conditions

The level of microbial activity inhibition correlated with the quantity of sodium azide added, with the strongest inhibition occurring at the highest spiked azide concentration (720 mg/g VSS). While ammonium was almost completely converted in the control samples, its conversion was increasingly hampered with increasing azide concentration based on the overall trend in these samples (Fig. 5.1a). The nitrate and nitrite profiles exhibited both ammonium and nitrite oxidation were inhibited upon addition of sodium azide to the sludge samples while nitrification occurred only in the control samples (Fig. 5.1g and h). This indicated inhibition of both ammonium oxidizing and nitrite oxidizing bacteria by the sodium azide. To eliminate the

CHAPTER 5

contribution of azide to soluble COD, the dissolved organic carbon (DOC) profile was monitored instead to quantify dissolved organic carbons in aqueous phase. DOC removal was similarly affected as the ammonium with highest removal in control samples (Fig. 5.1b). The measured DO was higher than 2 mg/L throughout the batch test (Fig. 5.1d) so the reduced microbial activities (ammonium and DOC removal) were not attributed to DO but associated with addition of the inhibitory sodium azide. However, DOC (heterotrophic) removal assays exhibited the microbial inhibition by sodium azide was incomplete even at sodium azide concentrations of 720 mg/gVSS. As regards the selective inhibition of nitrification in the sodium azide spiked samples, the reduction of ammonium in these samples (especially in 120 mgNaN₃/gVSS samples) was likely to be its utilization as a nitrogen source for the growth and metabolism of active heterotrophs and other microbial communities. The pH changes in the sludge samples upon addition of the sodium azide had been minimal and this was not expected to influence flocculation within the observed range (Fig. 5.1c). Extracellular potassium content was observed to have increased in the sodium azide spiked samples (Fig. 5.1f). This could indicate cell lysis as there was significant difference between intracellular and extracellular potassium concentrations (around 100 times concentration difference) [201].

CHAPTER 5

CHAPTER 5

Figure 5.1: Changes in (a) ammonium, (b) dissolved organic carbon, (c) pH, (d) DO, (e) supernatant turbidity, (f) potassium, (g) nitrate and (h) nitrite contents of SBR sludge samples subjected to various azide concentrations.

While supernatant turbidity of the control samples had decreased slightly over the test duration, supernatant turbidities of the azide spiked samples were higher and increased with increasing spiked sodium azide concentration (Fig 5.1e). This turbidity increase was irreversible in samples with azide concentrations of 720 mg/gVSS. The increasing

CHAPTER 5

supernatant turbidity indicated the occurrence of deflocculation in the sodium azide spiked samples because sodium azide alone as soluble solutes does not contribute to absorbance at 650 nm in the supernatant. The debris from cell lysis would contribute to increasing supernatant turbidity. The flocs were reduced in size and the proportion of small size flocs (<100 μm) increased with increasing sodium azide spiking concentration (Fig 5.2). The mean floc size and floc size distribution indicated occurrence of deflocculation, which was primarily due to erosion of components from the outer layer of the flocs and not the fragmentation of the flocs [148]. This erosion would have contributed to the higher proportion of smaller sized flocs and higher turbidity without causing a sharp decrease in the mean diameter. While sodium azide is known to inhibit microbial activities, it was not expected to have a significant impact on other major floc stabilizing interactions. This would explain the occurrence of floc erosion instead of floc structure fragmentation.

Figure 5.2: Measurement of (a) mean floc sizes, and (b) floc size distribution measured at the end of the batch test.

CHAPTER 5

Colorimetric measurements showed total SMP content had increased in the sodium azide spiked samples with proteins and humic acids making up much of this increase. The increase in SMP protein and other components was proportional to the concentration of sodium azide added. Increased SMP protein content is another reported indicator of deflocculation [15], and this supported the argument of there being deflocculation in the sodium azide spiked samples. The proteins and humic acids made up a major fraction of the LB and TB EPS based on the colorimetric and FEEM evaluations (Fig. 5.3a and b). The increased SMP protein content in sodium azide spiked samples was likely caused by erosion of bound proteins, especially the ones loosely attached (LB EPS) to the main floc body (Fig. 5.3a). Cell lysis was expected to have resulted in release of intracellular proteins into the SMP fraction, contributing to the SMP protein increase. The deflocculation in the sodium azide spiked samples was also expected to have contributed to the increase in SMP humic acids. This was exhibited by the decrease in loosely bound and tightly bound humic acid content (Fig. 5.3a). Decomposition of cell debris and lysis products could have also increased the humic acid content in the SMP fraction of deflocculated samples. The carbohydrate content decreased in the LB and TB EPS fractions of sodium azide spiked samples. This could be due to the inhibition of microbial metabolism resulting in reduced polysaccharide production or due to the break-up of bound polysaccharides from the floc body as a result of weakened floc interactions. Such break-ups of the bound EPS could be expected to have destabilized the floc body leading to subsequent deflocculation.

CHAPTER 5

Figure 5.3: Measurement of the SMP and bound EPS content by (a) colorimetry, (b) FEEM analysis at the end of the batch test. For the FEEM and synchronous fluorescence analysis, SMP, LB and TB EPS contents were measured at different voltages making only intra-fraction analysis feasible (PN-Protein, HA-Humic acid, AA-Amino acid, PS-Polysaccharides and FA-Fulvic acid).

CHAPTER 5

FEEM analysis, which provides more qualitative information on the EPS components, corroborated with the colorimetric analysis. There was increased Trp/Tyr containing proteins, humic acids (polycarboxylate and polyaromatic) and microbial products (polysaccharide-like) in the SMP fraction of sodium azide spiked samples (Fig. 5.3b). This increase in fluorescent EPS content was due to a combination of cell lysis leading to release of intracellular components and detachment of EPS from the floc body. The LB EPS fraction of all samples were rich in Trp/Tyr protein-like and amino acid-like components compared to the other EPS components. The Trp/Tyr protein and amino acid content were reduced in LB EPS fraction of sodium azide spiked samples, which could be due to their erosion from the main floc body (Fig. 5.3b). The TB EPS fraction of all samples were dominated by the Trp/Tyr protein-like components and amino-acid like components, especially in the sodium azide spiked samples where these components were even in higher amounts. This increase in TB EPS Trp/Tyr protein-like components of sodium azide samples was accompanied by an increase in microbial product-like or polysaccharide-like components along with a decrease in the polycarboxylate humic acid-like components. The decrease in polycarboxylate-like humic acid content, which was consistent with colorimetric measurements, could be due to deflocculation and the resulting detachment of bound humic acids. The increased Trp/Tyr protein-like, amino acid-like and microbial product-like components could be explained by the interaction of sodium azide with floc microbes and bound EPS, resulting in their increased extractability. The increased extractability of these fluorescence producing EPS components suggested a possibility of direct interaction between sodium azide and the bound EPS components as well. Such interactions could

CHAPTER 5

have also induced adverse effects on the floc stability and integrity, resulting in deflocculation.

5.3.2 Interactions between sodium azide and bound EPS

The intrinsic fluorescence corresponding to each EPS component can be used as a tool to assess possible interactions with a particular ligand - sodium azide in this case [188, 189]. Fluorescence quenching occurs upon interaction between sodium azide and the EPS components. Samples of extracted bound EPS (both TB and LB EPS) were exposed to various concentrations of sodium azide to assess the possibility of interactions between the components.

CHAPTER 5

Figure 5.4: The total fluorescent EPS intensities (a, b) and UV-absorption spectra (c, d) of TB EPS (a and c) and LB EPS (b and d) fractions interacted with different azide concentrations.

Fluorescence quenching was observed in all samples and in proportion to the amount of azide added, clearly indicating its interaction with the extracted EPS. In order to assess the quenching of EPS fluorescence by sodium azide, the Stern-Volmer equation was utilized [188, 189].

$$\frac{F_0}{F} = 1 + K_q T_0 [Q] = 1 + K_{sv} [Q]$$

where F_0 and F correspond to the EPS component fluorescence in the presence and absence of sodium azide respectively; $[Q]$ corresponds to the sodium azide concentration; K_q is the biomolecular quenching rate constant; T_0 corresponds to the average lifetime of the molecule in the absence of sodium azide quencher, which is 10^{-8} s in value [202], and the K_{sv} is the Stern-Volmer quenching constant.

CHAPTER 5

Fitting the data into the Stern-Volmer equation, the value of K_q for total EPS fluorescence quenching by the sodium azide was calculated to be 3.4×10^{10} ($R^2=0.997$) and 5.34×10^{10} ($R^2=0.988$) L/mol/s for TB EPS and LB EPS samples, respectively. These values are greater than the maximum diffusion collision quenching rate constant, 2×10^{10} L/mol/s [202], for quenchers interacting with biological macromolecules. The UV absorption spectra (Fig. 5.4c and d) of the EPS-azide complexes were different from the sum of the individual components (EPS and sodium azide) [189]. Such a change in the UV absorption spectra is only possible in the case of a static interaction between the fluorophore and the quencher [189]. These results support the argument there was occurrence of static quenching of EPS fluorescence by sodium azide, i.e. complexation of EPS-azide mixture [188, 189]. When considering individual EPS components (Fig. 5.4a and b) the fluorescence quenching was most significant for the Trp/Tyr amino acid-like and fulvic acid-like components in both TB and LB EPS samples with K_q values of around 1×10^{11} ($R^2=0.99$) for both the components.

5.3.3 Sodium azide induced deflocculation under low DO conditions

Deflocculation had been reported upon the addition of increasing amounts of sodium azide to the SBR sludge, which was speculated to be linked to the hampering of aerobic microbial activities [11]. Aerobic microbial activities can also be hampered under conditions of low DO (≤ 0.5 mg/L) and this has been cited as an important factor for the phenomenon of low DO deflocculation. However, recent studies have shown low DO related deflocculation is not a universal phenomenon. This was speculated to be due to the activities of facultative bacteria propagated through aerobic-anoxic

CHAPTER 5

cycling of sludge. These bacteria could survive and metabolize, including the production of EPS under such low DO conditions [198]. This could help compensate the reduced aerobic microbial activity and prevent deflocculation. Using a broad-spectrum inhibitor like sodium azide under low DO conditions will provide a more accurate assessment of the impact of facultative bacteria and overall microbial activity on biological flocculation. Sodium azide was added at 120 mg/gVSS as the SBR microbial inhibition (even though partial) occurred at this concentration and deflocculative symptoms were observed (Fig. 5.1e and Fig. 5.2a-b).

CHAPTER 5

Figure 5.5: Changes in the (a) supernatant turbidity, (b) potassium, (c) ammonium and (d) DO content of SBR sludge samples subjected to different batch test conditions (OX, AN and AZ-AN).

The supernatant turbidity of both the OX (high DO control) and AN (low DO control) samples were reduced from the initial sample turbidity (Fig. 5.5a). This suggested there was improved flocculation in these samples. However, this was not the case with the AZ-AN samples, where supernatant turbidity was significantly higher than the other set of samples and consistently increased throughout the batch test. This was indicative of active deflocculation in the AZ-AN samples. Analysis of the potassium results and the overall trends indicated higher cell lysis in the AZ-AN samples, which was expected based on earlier results (Fig. 5.1f). The mean floc size and the floc size distribution in the AN samples were comparable to the OX samples (Fig. 5.6). These results corroborate the argument there was lack of deflocculation in the AN (low DO control) samples. However, the flocs in the AZ-AN samples were smaller in size when considering the mean floc sizes of all the samples. Along with the reduction in mean

CHAPTER 5

floc size, the number of smaller sized flocs ($\leq 100 \mu\text{m}$) had also increased in the AZ-AN samples. The floc size distribution, increased number of very small sized flocs ($\leq 50 \mu\text{m}$), and the small reduction in mean floc size of AZ-AN samples indicated the occurrence of deflocculation through floc erosion.

Figure 5.6: Measurement of (a) mean floc sizes, and (b) floc size distribution measured at the end of the batch test.

Colorimetric evaluation of SMP content exhibited proteins and humic acids were highest in the AZ-AN samples. However, total SMP content in the OX and AN samples were negligible. The slight increase of SMPs in the AZ-AN samples suggested deflocculation in these samples. Evaluating the bound EPS content, TB EPS content were much higher than the LB EPS (Fig. 5.7a). Proteins and humic acids were observed to be the main components of the LB EPS fraction in all samples. As regards the TB EPS, the protein content of AZ-AN samples was lower while the humic acid content in these samples was higher than that of the OX and AN samples. The reduced protein levels along with the concomitant increase in humic acid suggested occurrence

CHAPTER 5

of hydrolytic degradation of proteins induced by the addition of sodium azide. Anoxic and anaerobic sludges have been reported to contain more hydrolytic proteins than aerobic sludges and they are mostly of intracellular origin [38]. Thus, the cell lysis like symptoms observed in the AZ-AN samples (Fig. 5.5b) could have led to the release of such hydrolytic enzymes of intracellular origin, and this could have led to the hydrolysis of bound proteins. The decomposition of the protein could have led to the increase in bound humic acid content. The carbohydrate content of AZ-AN samples was also lowest among all the samples, which could be due to reduced polysaccharide production in the inhibited samples or due to the deflocculation of these samples.

CHAPTER 5

Figure 5.7: Measurement of the SMP and bound EPS content by (a) colorimetry and (b) FEEM analysis at the end of the batch test. For the FEEM analysis, SMP, LB and TB EPS contents were measured at different voltages making only intra-fraction analysis feasible (PN-Protein, HA-Humic acid, AA-Amino acid, PS-Polysaccharides and FA-Fulvic acid).

FEEM analysis revealed results similar to Fig. 5.3b, the addition of sodium azide under low DO conditions resulted in increased fluorescent SMP content (Fig. 5.7b), especially the Trp/Tyr protein-like, corroborating the argument of occurrence of deflocculation in the samples. Again, the LB EPS of all samples were especially rich in Trp/Tyr protein-like and amino acid-like components, while the TB EPS mainly consisted of protein-like and polycarboxylate humic acid-like components (Fig. 5.7b). Similar to Fig. 5.3b, higher amounts of Trp/Tyr protein-like, amino acid-like and microbial product or polysaccharide-like components were extracted in TB EPS fraction of the azide spiked (AZ-AN) samples (Fig. 5.7b). The extractability of Trp/Tyr protein-like and other fluorescence producing EPS components in sludge flocs

CHAPTER 5

was thus enhanced by the sodium azide addition. This increased extractability of specific EPS components regardless of DO conditions was likely due to interactions between sodium azide and floc components, including bound EPS.

5.4 Discussions

Sodium azide is an inhibitor of ATP synthesis [11, 203]. However, the amount of sodium azide required to trigger complete sludge microbial inhibition is still not well known [200, 204]. Studies have reported incomplete microbial inhibition, in terms of biological COD and ammonium removal, at sodium azide concentrations as high as 720 mg/gTSS [200]. One also has to consider the possibility of cell lysis at very high azide concentrations [205]. It was thus necessary to experimentally assess the microbial inhibitory and cell lysis effects of different sodium azide quantities on the SBR sludge in this study. This allowed for better assessment of the impact of increasing microbial inhibition on the sludge floc stability and integrity.

The SBR sludge expectedly remained well flocculated under conditions of high DO and in the absence any other stress - i.e. sodium azide influenced microbial inhibition. This was observed in the authors' previous work [199] and has precedent [11, 15]. The maintenance of floc stability in the sludge samples could be explained by the promotion of aerobic metabolic activities in the control samples, as reported in the literature [11]. These activities include increased EPS production, especially under high DO conditions, which can be expected to help stabilize the floc matrix [38, 64, 194]. Additionally, the SBR sludge did not deflocculate under low DO conditions, as was noted in the authors' previous work. This is contrary to reports in the literature [15]. The absence of deflocculation was attributed to the type of microbial

CHAPTER 5

communities cultured in the SBR. Facultative bacteria are enriched during the anoxic reaction phase of the SBR and these bacteria can tolerate extended low DO conditions unlike obligate aerobes [206]. The facultative bacteria can metabolize under such conditions and are even known to be able to produce EPS [198], which will be important in stabilizing the floc structure and increasing its resistance to deflocculative stresses. The activities of such facultative bacteria were thus speculated to be responsible for the absence of low DO deflocculation observed in this study. The contrasting reports on low DO deflocculation could be due to the varying sludge conditioning measures leading to differences in the microbial communities cultivated, i.e. more obligate aerobes or a mixture of aerobes and facultative bacteria.

When sodium azide was added, microbial activities were inhibited (indicated by reduced ammonium and biological DOC removal). Sodium azide has been reported to inhibit nitrification [207], and so would explain the reduced conversion of ammonium to nitrates with increasing azide concentration. More importantly, the reduced ammonium and biological COD removal are symptomatic of an overall reduction in microbial growth and activities, which are essential in maintenance of the flocs [203, 207]. EPS production has been reported to begin as early as in the microbial growth phase [198, 208, 209]. However, with inhibition of microbial growth by sodium azide [203], the EPS production can be adversely affected as well and this will reduce flocculation rates and floc stability. The cell lysis like symptoms (extracellular potassium content) in sodium azide spiked samples could have been due to increased reactive oxygen species (ROS) induced damages brought about by the inhibition of bacterial peroxidase and catalase enzymes, which would have otherwise counteracted

CHAPTER 5

the ROS produced as a by-product of oxidative reactions [210-212]. The inhibition of microbial growth and metabolic activities, especially at very high azide concentrations (≥ 100 ppm), was thus reasoned to be responsible for the deflocculation in the azide spiked samples. Sodium azide can selectively inhibit nitrate reductase, which will be required by the facultative bacteria to utilize nitrate as an electron acceptor [213-215]. With the inhibition of these enzymes by sodium azide, anoxic respiration and metabolism of the facultative bacteria would have been compromised and this would leave them unable to compensate for the reduced aerobic microbial activity [216]. This was expected to have led to the deflocculation in the sodium azide spiked samples under low DO conditions.

Sodium azide is highly reactive compound that can interact with other components such as ferric ions in the floc ecosystem [12]. In this study, sodium azide was observed to have had an impact on the type of tightly bound EPS extracted. The extraction protocol for the TB EPS was such that the extracted EPS composition would be consistent for replicate samples. Significant changes in the extracted TB EPS quality and quantity would only be due to the additional influence of one or more components in the sample [217]. In this case, the significantly increased levels of Trp and Tyr containing proteins in the TB EPS fraction of azide spiked samples indicated interaction between sodium azide and the floc EPS. Fluorescence quenching studies between extracted EPS and the sodium azide further confirmed this, especially with the Trp and Tyr amino acid residues. LB EPS has been reported to protect the floc body by interacting with toxic compounds and trapping them before the floc core was affected [127, 128]. The same could have occurred in this study, with the toxic effects

CHAPTER 5

from spiked sodium azide being mitigated by the bound EPS (both LB and TB). However, above a threshold concentration, the interactions of sodium azide with floc EPS (especially proteins) and bacteria could be expected to adversely affect floc integrity and increased the propensity for the sludge to deflocculate. Such a floc destabilizing mechanism by sodium azide should also be considered with regards to the sludge deflocculation observed in this study.

5.5 Conclusions

The SBR sludge remained well flocculated under both high and low DO conditions but was so only in the absence of a stressor. This was contrary to the commonly reported deflocculation associated with low DO conditions. At high concentrations of sodium azide, the sludge microbial activities were inhibited, and cell lysis was observed. The concurrent deflocculation in these samples indicated the importance of microbial activities and viability in maintenance of sludge flocs and mitigation of sludge deflocculation. The sodium azide induced deflocculation under low DO conditions indicated facultative bacteria activity, cultivated under alternating “with and without” aeration conditions, could play a larger role in the maintenance of sludge flocs than previously considered. The facultative bacteria in the sludge flocs compensated for reduction in activities of aerobes under low DO conditions. When their activities were also compromised (sodium azide based inhibition), it led to deflocculation. Thus, biological flocculation level in a system would be influenced by overall microbial activities and not just that of the aerobes and anaerobes.

CHAPTER 6

CHAPTER 6

Impact of cation chelation on destabilization of the sludge floc ecosystem

6.1 Introduction

The involvement of bacterial communities, including facultative bacteria, in the maintenance of SBR sludge flocs and mitigation of deflocculation has been recently reported [199]. However, the microbes are but one of the factors influencing floc stability and integrity. The extracellular polymeric substances (EPS) produced by microbes make up a majority of the floc ecosystem and they perform a vital role in stabilizing the floc structures through their interactions [193]. The EPS along with the microbes possess negatively charged surfaces, especially in the pH range typically observed in the activated sludge processes [218]. This property of the EPS and microbial surfaces allows for ionic interactions with positively charged cations [194], which helps with formation and stabilization of the floc matrix. The type and valency of cations interacting with the EPS can influence strength of these interactions, with multivalent cations (Fe^{3+} , Al^{3+} , Ca^{2+} , Mg^{2+} , Fe^{2+}) promoting stronger interactions than their monovalent counterparts (Na^+ , K^+) [17, 18]. This is due to the increased number of interactions possible with increasing cation valencies. Increases in the ratio of monovalent to multivalent cations has been reported to reduce floc strength and increase the propensity for sludge deflocculation [74]. Microbial reduction of Fe^{3+} to Fe^{2+} as a by-product of anoxic respiration has been reported to promote sludge deflocculation [11]. The divalent cations, such as magnesium and calcium, are also known to interact with membrane proteins and lipopolysaccharides in the outer membranes of bacteria (Gram-negative). This helps stabilize the outer layer and protect the bacterial cell from stresses [219, 220]. As regards the importance of

CHAPTER 6

multivalent cations, factors that affect floc cationic content can be expected to influence the floc ecosystem (microbes and cationic interactions) more acutely.

Similarly, sequestration of divalent calcium and magnesium by a chelating agent like ethylenediaminetetraacetic acid (EDTA) will affect the formation of well settling sludge flocs due to reduced ionic interactions [70]. The EDTA can complex strongly with metals and cations, which will not only sequester the cations from the floc body but also shield them from further interactions. This can lead to irreversible deflocculation while EDTA are present in the system. There have been reports for this type of deflocculation due to the sequestering of cations by EGTA [176]. EDTA has also been reported to induce anti-bacterial effects on planktonic bacteria and bacterial communities in biofilms [219, 220].

This study had been undertaken to evaluate the effects of non-complexed EDTA and calcium complexed EDTA on SBR sludge flocs. While the non-complexed EDTA could exhibit cation chelation tendencies, pre-complexation with calcium ions was expected to counteract this tendency. This will then help elucidate the varying effects of cation chelation on stability and integrity of the floc structure. There are reports in the literature on the effect of EDTA on stability of sludge flocs [70, 221]. However, these had argued for occurrence of deflocculation largely based on increases in supernatant turbidity only, which is not an accurate indicator of the deflocculation phenomenon. They had also not considered the impact of EDTA on the viability and activities of floc microbes, which can have an additional influence on the biological flocculation process [11]. This work was thus designed to better elucidate the impact

CHAPTER 6

of cation chelation by EDTA on the microbial communities, their activities and the overall floc stability.

6.2 Materials and Methods

6.2.1 Sludge samples

The experiments were performed with sludge samples collected from a laboratory scale 8-litre sequencing batch reactor (SBR) fed with synthetic wastewater, as specified in section 4.2.1 of Chapter 4.

6.2.2 Experimental protocols

The sludge samples were dosed with various EDTA concentrations: i.e. 0, 90, 180 and 270 mg/gVSS and after which the samples were subjected to aerobic conditions ($\text{DO} \geq 1$ mg/L). The tests were performed in duplicates with the sludge conditioning and analysis performed at 25 °C, unless otherwise specified. Eight samples of 100 mL each SBR sludge with volatile suspended solids (VSS) of 2 g/L, were placed in 100 mL bottles. The sludge was collected at the end of the aerobic react phase, settled for 30 minutes after which 50 mL of the supernatant was discarded leaving behind 50 mL of settled sludge. The discarded supernatant was replaced with SBR fresh feed and EDTA solution. The latter was drawn from a 37.2 g/L stock EDTA disodium dihydrate solution to bring about the required EDTA concentrations. All the samples were subjected to “zero-hour” soluble COD, DO, pH, ammonium, potassium and turbidity measurements. Subsequently, the samples were shaken at 190 rpm in open bottles to allow for diffusion of oxygen from the air into the samples to promote aerobic conditions.

CHAPTER 6

To assess the impact of calcium-EDTA complexation on sludge flocs, EDTA solution (from a 37.2 g/L EDTA disodium salt dihydrate stock solution) was first mixed with calcium chloride solution such that the ratio of EDTA to calcium ions in the mixture was 10:1. This solution was then shaken for an hour at 250 rpm to allow for effective complexation (Ca-EDTA). After the complexation, one set of sludge samples was interacted with non-complexed EDTA while another set of samples was interacted with Ca-complexed EDTA, with the final EDTA concentrations in all the samples being 270 mg/gVSS. 6 samples of 100 mL, each with volatile suspended solids (VSS) of 2 g/L, were placed in 100 mL bottles. The SBR sludge was collected after the aerobic react phase, settled for 30 minutes after which 50 mL of the supernatant was discarded. The discarded supernatant was replaced with the SBR fresh feed and either EDTA or Ca-EDTA at a concentration of 270 mg/gVSS. The sludge samples were then shaken in open bottles at 190 rpm to promote aerobic conditions. The experimental conditions were tested in duplicates and at 25 °C.

Measurements were made at regular intervals (1.5 or 2 hours). DO, pH, potassium and ammonium readings were measured using electrochemical probes. 2 mL of sludge samples were collected for soluble COD measurements. All the samples were then mixed by hand and left to stand undisturbed for 10 minutes, after which 1.5 mL of the supernatant was collected for turbidity measurements. After the turbidity measurements, the supernatant samples were returned to the respective sample bottles and the sample bottles were then returned to the shaker for open bottle shaking at 190 rpm. The floc size measurements and bound EPS extraction were performed at the end of the batch test in order to assess the effects of EDTA on the flocs and bound EPS content.

CHAPTER 6

6.2.3 Analytical Methods

TSS, VSS, soluble COD, DO, pH, ammonium concentration, potassium concentration and floc sizes were measured as specified in Chapter 3. The supernatant turbidity was measured with a UV-Spectrophotometer and expressed in terms of absorbance at 650 nm. The mean floc size and floc size distribution profiles were utilized to express the changes in the floc sizes among samples. The SMP and bound EPS were extracted by the mild heat extraction protocol as specified in Chapter 3. The colorimetric measurements of the EPS fractions were performed with the modified Lowry's method (for proteins and humic acids) and the phenol-sulphuric acid method (for carbohydrates) [133, 185]. The FEEM and synchronous fluorescence analyses were performed in accordance with the methods specified in Chapter 3. The calcium (Ca) and magnesium (Mg) contents in the various EPS fractions were measured with a Microwave Plasma Atomic Emission Spectrometer (Agilent, USA). The samples were filtered with 0.45 μm pore size filter and were acidified with 1% HNO_3 prior to the measurement. This was done in order to remove the effect of pH on the ionic content measurements.

6.3 Results

6.3.1 Influence of EDTA on floc stability and integrity

Supernatant turbidity of the control samples was lower than the EDTA spiked samples throughout the batch test. In samples spiked with 180 mgEDTA/gVSS or higher, the supernatant turbidity was not only higher but also increasing throughout the batch test (Fig. 6.1a). The increase in supernatant turbidity was especially significant in samples spiked with 270 mgEDTA/gVSS, with the supernatant turbidity being 10 times that of

CHAPTER 6

the control samples. The colorimetric measurement of SMP fractions exhibited significantly increased protein, humic acid and polysaccharide content in the EDTA spiked samples (Fig. 6.2). This was corroborated by the FEEM analysis profile, which exhibited a similar increase in the fluorescence intensities of different EPS components in the SMP fraction in these samples. EDTA did not exhibit natural fluorescence in the excitation/emission wavelength range and thus the changes in fluorescence can be attributed to the EPS components alone. The increased supernatant turbidity and SMP content in the EDTA spiked samples were observed in tandem with a decrease in the mean floc sizes by the end of the batch test (Fig. 6.1b and c). The floc size decrease was most prominent in samples spiked with an EDTA concentration greater than or equal to 180 mg/gVSS (Fig. 6.1b). Along with the mean floc size decrease, the floc size distribution profile in these samples consisted of an increase in the number of smaller flocs (over a range of 0-400 μm) coupled with a marked decrease in the number of larger flocs (400-800 μm) (Fig. 6.1c). These results indicated occurrence of both significant floc erosion and fragmentation in samples spiked with EDTA at a concentration of 180 mg/gVSS or higher.

CHAPTER 6

Figure 6.1: Profiles of (a) supernatant turbidity changes, (b) mean floc size and (c) floc size distribution of samples subjected to different EDTA concentrations. The mean floc size and floc size distribution measurements were performed at the end of the batch test.

The LB EPS fractions was mainly composed of proteins, particularly Trp/Tyr containing proteins (Fig. 6.2a-b). The extracted TB EPS was of a similar composition to the LB EPS in terms of the high protein content (including Trp/Tyr containing proteins) and humic acids (Fig. 6.2a-e). In the EDTA spiked samples with noticeable deflocculation (180 mgEDTA/gVSS or higher), the humic acid content was significantly reduced in both LB and TB EPS fractions while the reduction in protein and polysaccharide content was less pronounced. However, FEEM analysis exhibited reduced fluorescence corresponding to bound EPS proteins, polysaccharides and humic acid in the deflocculated samples. The synchronous fluorescence analysis specifically tracks fluorescence corresponding to the tryptophan (Trp) residues in the system, with the Trp residues exhibiting a characteristic peak between an excitation

CHAPTER 6

wavelength of 280-295 nm (Fig. 6.2c-e). This wide range in the characteristic peak of Trp residues is dependent on the microenvironment around the Trp residues (hydrophilic or hydrophobic). Thus, factors like EPS extraction procedures can affect the Trp fluorescent peak, which was the main reason for the red shift of Trp peak (290 nm) in the TB EPS fractions (Fig. 6.2e). The synchronous fluorescence analysis also exhibited increased fluorescence of Trp residues in the SMP fractions along with Trp fluorescence decrease in the bound EPS fractions of deflocculated samples (180 mgEDTA/gVSS or higher) (Fig. 6.2c). The synchronous fluorescence profile of LB EPS fractions did not indicate a similar trend, while the profile of TB EPS fractions exhibited a slight blue shift in the characteristic Trp peak (Fig. 6.2d and e). This could be due to the interactive influence of EDTA on the sludge flocs. The distribution of EPS (including fluorescing components) across the SMP and bound EPS fractions of deflocculated samples indicated the solubilization of bound EPS upon interaction with sufficient quantities of EDTA. This solubilization of EPS was indicative of reduced interactions in the floc body, which was partly responsible for the deflocculation.

CHAPTER 6

CHAPTER 6

Figure 6.2: Measurement of chemical composition of EPS by (a) colorimetry, (b) FEEM analysis and (c, d and e) synchronous fluorescence analysis (with a $\Delta\lambda = 60$ nm) in the various EPS fractions.

Microbial activities, in terms of biological ammonium and COD removal, were most efficient in the control samples (Fig. 6.3a and b). The adverse effects on ammonium removal was most evident at EDTA spikes of 180 mg/gVSS or higher, with the ammonium content increasing over the test duration (Fig. 6.3a). This indicated the

CHAPTER 6

EDTA spiking hampered the activities of ammonium oxidizing bacteria at a certain critical EDTA concentration. The increased ammonium content could also be due to the degradation of protein and amino acid residues (Fig. 6.2a-e) released into the bulk solution as a result of the deflocculation. The nitrate profile exhibited a trend similar to the ammonium profile in that nitrate production was hampered at an EDTA spike of 180 mg/gVSS (Fig. 6.3c). The nitrate profile also exhibited a reduction of nitrate content in the samples spiked with 270 mgEDTA/gVSS over the test duration. The observation could be due to the activity of facultative denitrifying bacterial communities, which can utilize nitrate even under higher DO conditions through certain pathways [222, 223]. These bacteria are enriched by intermittent aeration conditions [222], which can explain their presence in the cyclically aerobic-anoxic conditioned SBR sludge flocs used in this study. While ammonium oxidizing bacteria and heterotrophs were hampered by the EDTA spike, the facultative denitrifying bacteria could have still been active, utilizing nitrate for their respiration and energy production. The soluble COD removal was increasingly hampered with the EDTA spiking (Fig. 6.3b). While COD removal was still observed in samples spiked with 90 mgEDTA/gVSS, higher spikes of EDTA resulted in increasing residual COD over the test duration. This was due to the deflocculation in these samples. The COD profile indicated the EDTA spiking had inhibited and hampered heterotrophic bacterial activity at these critical EDTA concentrations. The COD profile also suggested the inability of SBR bacterial communities in degrading the EDTA. The increasing extracellular potassium content in the samples indicated the occurrence of cell lysis (Fig. 6.3d) [201]. Thus, the spiking of sludge samples with sufficient quantities of

CHAPTER 6

EDTA adversely affected microbial viabilities and hence their activities, and this was a factor in the promotion of deflocculation.

CHAPTER 6

Figure 6.3: Profiles of (a) ammonium, (b) soluble COD, (c) nitrate, (d) potassium, (e) calcium, (e) magnesium and (e) iron content of samples subjected to various EDTA concentrations. The cationic content in the different EPS fractions were assessed at the end of the batch test.

The evaluation of the calcium (Ca^{2+}) and magnesium (Mg^{2+}) content in the different EPS fractions revealed magnesium to be more prevalent in the TB EPS of the sludge samples while calcium was mainly in the SMP fraction (Fig. 6.3e). This indicated the magnesium ions were incorporated more into the floc body to help stabilize the SBR sludge flocs. The long-term conditioning of SBR sludge flocs was such that the divalent cations were present in much higher amounts than Fe^{3+} ions. The reduction of divalent cations in the TB EPS fractions of EDTA spiked samples was expected to be due to their chelation by the added EDTA, especially at 180 mgEDTA/gVSS or higher. This chelation was attributed to higher binding affinity of the divalent cations for EDTA molecules when compared to the EPS [224]. The chelation of divalent

CHAPTER 6

cations would reduce ionic interactions in the floc body and enhance the solubilization of bound EPS (Fig. 6.2). The complexation of unbound calcium and magnesium ions with EDTA would also make them unavailable for further interactions with the EPS content and floc body, further leading to destabilization of the floc structure and deflocculation.

6.3.2 Influence of calcium complexed EDTA on the sludge flocs

The supernatant turbidity in the Ca-EDTA spiked samples was significantly lower (10 times) than in the samples interacted with non-complexed EDTA (Fig. 6.4a) and was comparable to that of the control samples. The flocs in the EDTA spiked samples were also smaller in size in comparison to the flocs in the Ca-EDTA spiked and control samples (Fig. 6.4b). The floc size distribution profile exhibited a significant increase in the number of smaller sized flocs (0-100 μm) in the EDTA spiked samples (Fig. 6.4c). The total SMP content was significantly increased in the samples spiked with non-complexed EDTA (Fig. 6.5a-c). This increase was mainly from the increase in proteins, including the Trp and Tyr containing proteins (Fig. 6.5a-c). In contrast, the SMP protein and humic acid contents were very low in the Ca-EDTA spiked samples and this was comparable to the control samples. The higher carbohydrate content in the Ca-EDTA spiked samples was likely from the colorimetric measurement of EDTA and its complexes rather than floc deflocculation. The increase in supernatant turbidity and total SMP content coupled with floc size decrease in the EDTA spiked samples was consistent with the earlier results (Fig. 6.1 and 6.2). This further confirmed the deflocculative influence of EDTA at 270 mgEDTA/gVSS. However, complexation of EDTA with calcium ions prior to its interaction with the sludge samples significantly

CHAPTER 6

reduced its propensity to induce sludge deflocculation. This indicated sludge deflocculation by non-complexed EDTA was mainly influenced by chelation of cations from the floc body, further corroborating the importance of ionic interactions in the stability and integrity of sludge flocs.

Figure 6.4: Profiles of (a) supernatant turbidity changes, (b) mean floc size and (c) floc size distribution of samples subjected to non-complexed and Ca-complexed EDTA

CHAPTER 6

additions. The mean floc size and floc size distribution measurements were performed at the end of the batch test.

The LB EPS content was reduced in both the EDTA and Ca-EDTA spiked samples, as exhibited by the colorimetric and FEEM analysis. The interactions of EDTA with the cationic interactions in the outer layers of the floc was expected to have led to the disruption and dissociation of LB EPS from the main body. The release of LB EPS was not expected to have adversely affected the floc integrity as much as the observed reduction in TB EPS content. Such a reduction in the TB EPS content was only observed in the EDTA spiked samples (Fig. 6.5a-e). This together with the increased SMP content in these samples indicated the solubilization of bound EPS. The chelation of divalent cations would have disrupted their interactions with the EPS components leading to bound EPS solubilization and deflocculation. The complexation of EDTA with calcium ions reduced its tendency to chelate the cations present in the floc body. This helped preserve the cationic interactions (cation bridging and ionic interactions) with the bound EPS and prevented the occurrence of deflocculation.

CHAPTER 6

CHAPTER 6

Figure 6.5: Measurement of chemical composition of EPS by (a) colorimetry, (b) FEEM analysis and (c, d and e) synchronous fluorescence analysis (with a $\Delta\lambda = 60$ nm) in the various EPS fractions.

The ammonium and soluble COD profiles exhibited ammonium and soluble COD removal was severely hampered in the non-complexed EDTA spiked samples (Fig. 6.6a and b). The reduced nitrate content in these samples, expected based on earlier results (Fig. 6.3c), was due to the utilization of nitrates by the facultative denitrifying

CHAPTER 6

bacteria (Fig. 6.6c) [222, 223]. These results contrasted the samples spiked with Ca-EDTA, where ammonium removal and nitrate production were comparable to the control samples. This indicated the complexation of EDTA with calcium reduced its propensity to inhibit bacterial activities leading to the greater nitrification activities under high DO conditions. The soluble COD profile of Ca-EDTA spiked samples exhibited the incomplete COD removal in these samples. This was more to be due more to the microbes' inability to effectively degrade the complex EDTA molecules rather than any adverse impact on microbial viability and activity. There is precedent for poor biodegradability of non-complexed EDTA [220]. The bacteria in these samples (containing Ca-EDTA) could be reasoned to have prioritized the utilization of more easily degradable COD, i.e. glucose, over the more complex EDTA. This could better explain the soluble COD profile of Ca-EDTA spiked samples. The extracellular potassium content was observed in increasing amounts only in the EDTA spiked samples, suggesting occurrence of cell lysis in only these samples (Fig. 6.6d). These results indicated the complexation of EDTA with calcium ions counteracted their anti-bacterial and inhibitory effects. This in turn prevented the occurrence of deflocculation in Ca-EDTA spiked samples. Divalent cations, therefore, had a role in the maintenance of microbial viability, likely in stabilization of bacterial cell membranes and in general microbial metabolism, which when disrupted resulted in microbial activities hampering effects. This should be an important consideration when evaluating the importance of divalent cations in the overall biological flocculation process.

CHAPTER 6

CHAPTER 6

Figure 6.6: Profiles of (a) ammonium, (b) soluble COD, (c) nitrate, (d) potassium, (e) calcium, and magnesium content of non-complexed EDTA and Ca-EDTA spiked samples. The cationic content in the bound EPS fractions were assessed at the end of the batch test.

The cation content distribution profile also exhibited the contrasting effects of EDTA and Ca-EDTA spiking on cationic content in the extracted bound EPS fractions (Fig. 6.6e). The calcium (Ca^{2+}) and magnesium (Mg^{2+}) content were significantly reduced in the samples spiked with non-complexed EDTA, especially in the TB EPS fraction. This was due to their chelation by EDTA and was symptomatic of the deflocculation. This was not the case in the samples spiked with Ca-EDTA, where cation content distribution was comparable to the control samples further corroborating the lack of deflocculation in these samples. The increased calcium content in the Ca-EDTA spiked samples was not expected to have promoted chemical flocculation through cationic bridging. This was rather due to its strong complexation with EDTA, which

CHAPTER 6

would leave it unavailable for interactions with the floc body. This was reflected by the lack of increase in the calcium content in the bound EPS fraction of Ca-EDTA spiked samples. Pre-complexation of EDTA with calcium reduced the cation chelating and sequestering tendencies of EDTA, which was a major reason for the floc maintenance and mitigation of deflocculation.

6.4 Discussions

The spiking of EDTA had an adverse influence on the stability of sludge flocs after a certain critical threshold concentration (180 mgEDTA/gVSS or 360 mgEDTA/L) and promoted deflocculation in terms of both floc erosion and floc fragmentation. There is precedent for this in the reported literature. Eriksson and Alm [70] reported increased residual turbidity and SMP content in sludge samples added with 200 mg/L of EDTA or more. Sheng, Yu [221] reported the addition of EDTA at concentrations between 292 and 1168 mg/L to aerobic and anaerobic flocs resulted in their increased shear sensitivity and deflocculation. This was evaluated mainly based on the increased “equilibrium dispersed mass concentration”, which was characterized from increased supernatant turbidity in the reported deflocculated samples. They reported anaerobic flocs were more sensitive to the EDTA spike, with deflocculative symptoms occurring at a spike of 292 mg/L, than the aerobic flocs which exhibited such symptoms only at a spike of 876 mg/L or higher. It has to be noted the dispersed mass concentration was calculated from the supernatant turbidity values and changes in floc size were not monitored. Sheng, Yu [221] speculated the ionic strength increase contributed by the Na^+ and $[\text{H}_2\text{EDTA}]^{2-}$ ions led to the compression of electrical double layer inducing a stabilizing effect on the flocs. However, beyond a critical EDTA concentration, the

CHAPTER 6

chelating effects of EDTA were speculated to have become the dominant phenomenon leading to floc destabilization and subsequent deflocculation [221].

The electrical double layer compression phenomenon could explain the lack of significant deflocculation in the samples spiked with 90 mgEDTA/gVSS (or 180 mg/L) while cation chelation became more prominent at higher EDTA spikes. The increased cation chelation adversely influenced the floc interactions in the floc body destabilizing the sludge flocs and promoted deflocculation. The cation chelation phenomenon was further confirmed to play a major role in the EDTA influenced deflocculation when pre-complexed Ca-EDTA did not have the same deflocculative influence as their non-complexed counterparts. The mitigation of deflocculation was not brought about by chemical flocculation but by the reduction in cation chelation tendencies of EDTA.

EDTA did not only influence the floc stability through the disruption of ionic interactions, but also through their impact on the floc microbial communities. Reports in literature have stated EDTA possessed antibacterial and antimicrobial effects [219, 225]. This has been utilized in health care to disrupt biofilms and act as antiseptics. EDTA's mode of antimicrobial action has been reported to involve the chelation of divalent cations, Mg^{2+} and Ca^{2+} , that stabilize the outer membrane structures of bacterial cells through their involvement with membrane proteins and polysaccharides [219]. This could explain the hampered autotrophic and heterotrophic microbial activities and increased cell lysis (extracellular potassium content) in the samples spiked with 180 mgEDTA/gVSS or higher, which also coincided with increased sequestration of cations from the floc body. This disruption to microbial viability and

CHAPTER 6

their activities could be expected to have affected their other metabolic activities including EPS production. Considering the importance of microbial activities in floc maintenance, their disruption could be expected to have contributed to the floc destabilization and subsequent deflocculation. The pre-complexation of EDTA with calcium reduced its propensity for cation chelation and it was also observed to have counteracted its negative effect on microbial viability and activities. The autotrophs and heterotrophs functioned more effectively in the Ca-EDTA spiked samples while not exhibiting symptoms of cell lysis or structural destabilization. This further confirmed the wide-ranging floc destabilizing effects of divalent cation chelation, which can adversely impact the overall floc ecosystem more acutely. These results confirmed divalent cations play a more important role in the biological floc ecosystem than previously considered, which can also severely affect the liquid-solids separation process.

6.5 Conclusions

The spiking of SBR sludge samples with EDTA at concentrations of 180 mg/gVSS or higher, resulted in significantly hampered microbial activity and viability, indicated by reduced ammonium and biological COD removal along with increased extracellular potassium content. Additionally, the calcium and magnesium contents in the SMP fraction increased significantly while decreasing in the bound EPS fraction of deflocculated samples. Deflocculation was confirmed in these EDTA spiked samples in terms of increased supernatant turbidity and SMP content along with a decrease in the mean floc size. The floc size distribution profile indicated the occurrence of both erosion and fragmentation of flocs in the deflocculated samples. Cation chelation by EDTA was reasoned to have been responsible for weakened interactions leading to the

CHAPTER 6

deflocculation event. When EDTA was pre-complexed with calcium ions, the adverse effects on microbial communities and the cation chelating effects were mitigated and deflocculation was not observed. The results confirmed the role of multivalent cations in not only stabilizing the floc structure through cation bridging, but also in the maintenance of viability of microbes in the floc ecosystem. The disruption of their balance was a significant influence on break-up of the floc structure and stability.

CHAPTER 7

CHAPTER 7

Overall Conclusions and Practical Implications

7.1 Absence of deflocculation under low DO conditions and its practical implications

Deflocculation under low DO conditions (≤ 0.5 mg/L) has been proven to not be a universal phenomenon regardless of the reduced aerobic microbial activity in the sludge samples, which contrasted the presently available literature. The absence of deflocculation was confirmed based on the insignificant turbidity, SMP and floc size changes when exposed to low DO conditions. This type of an evaluation is more holistic and accurate than previous reports which have diagnosed deflocculation based on supernatant turbidity alone. The evaluation of floc size changes in this dissertation studies has also allowed for the differentiation of experimental conditions (low DO, sodium azide spiked or EDTA spiked) impacting on the planktonic bacterial communities from the floccular aggregates. The experimental methods and parameters used to evaluate biological flocculation process must be considered carefully to perform more accurate evaluations owing to the complexities of sludge and floc ecosystems.

The reasons for the observed absence of deflocculation under low DO conditions can be attributed to the microbial populations in the flocs, which are influenced by the long-term DO conditions. This in turn will affect its reaction to immediate and short-term transients in DO. The sludge samples utilized in other studies were reportedly conditioned under more aerobic conditions, which can be expected to promote the growth and metabolism of more aerobes in the sludge flocs [12, 14]. This led to a

CHAPTER 7

more prominent effect of aerobes on the floc integrity under low DO conditions (< 0.5 mg/L) in the reported studies. This was not the case with the sludge samples tested in this dissertation studies, which were conditioned under more cyclic aerobic-anoxic conditions. The cyclic (aerobic-anoxic) conditioning of sludge samples promoted facultative bacteria which can metabolize under low DO conditions, unlike obligate aerobes that require consistent aerobic conditions to be viable and active. The facultative bacterial communities cultivated under the cyclic conditions played a role in maintaining floc integrity and mitigating deflocculation under low DO conditions. The high bound EPS protein content of the sludge flocs was another factor in promoting higher floc stability, integrity and strength, which helped to resist deflocculation under low DO conditions. Deflocculation was only observed when the microbial communities were inhibited using sodium azide, a mitochondrial uncoupler and an inhibitor of nitrate reductase enzymes. This further elucidated the direct and indirect impact of microbial communities and their activities as a whole on the maintenance of floc stability and integrity. The immediate and short-term DO transients do not influence the biological flocculation and floc maintenance as much as the overall microbial communities cultivated over the long-term and their metabolic activities.

These results have practical significance as well. In WWTPs, the control of DO at a particular level will not always yield effective biological flocculation nor will short term transients in DO bring about rapid adverse effects on the flocs. This is an important consideration in cases where excess DO is supplied with the intention of inducing and maintaining higher amounts of biological flocculation. This is not energy

CHAPTER 7

effective nor cost-effective considering the high costs of aeration. Cyclic aerobic-anoxic conditioned sludges can be reasoned, based on this dissertation studies, to yield flocs of similar integrity and stability to more aerobically conditioned sludge flocs owing to the compensatory impact of facultative bacterial communities. Anoxic-oxic systems are utilized in many wastewater treatment processes due to their multipurpose use in complete nitrogen removal and COD degradation and based on the results in this thesis this type of strategy would not compromise the stability and integrity of floccular or granular aggregates in the system.

However, more studies must be performed to establish the feasibility of such a strategy on a larger scale like in WWTPs. This is because the sludge samples in WWTPs are exposed to much more complexities, including in the influent wastewater composition, environmental conditions and process disturbances they are exposed to, which can influence the microbial community distribution and their activities more sharply. The higher number of variables in WWTPs must be considered more holistically while developing the strategies to predict and mitigate floc disruption and deflocculation more effectively. One possible mechanism for the prediction of floc structural condition or deflocculation can be through the monitoring of metabolites and microbial by-products, like proteins, in the bulk fraction of treated wastewater. Metabolomic and proteomic based studies can be utilized to identify the link between the metabolite/protein content in the bulk fraction of treated wastewater and the floc structural conditions (stable or disrupted). The detailed knowledge acquired from that study could then be assessed for its replicability at a larger scale, which should then

CHAPTER 7

allow for the development of more effective strategies to monitor the stability and integrity of sludge flocs or aggregates cultivated in the system.

Additionally, the multivalent cations and their interactions were established to have played a role in the stabilization of the sludge flocs under low DO conditions. The valency of cations has been reported to be directly proportional to the floc stability and thus the ionic form of iron ($\text{Fe}^{3+}/\text{Fe}^{2+}$) in the system can have varying influence on the floc strength and integrity [14]. The sludge samples in this dissertation studies were not conditioned with excess iron and thus the oxido-reductive effects of iron, especially under low DO conditions, were not speculated to have influenced floc stability and integrity as reported in literature. However, upon the sequestering of divalent calcium and magnesium ions from the floc body by EDTA, bound EPS solubilization and deflocculation occurred due to reduced ionic interactions in the floc body. Pre-complexation of EDTA with calcium ions counteracted its deflocculative tendencies confirming multivalent cation chelation to be the main reason for the promotion of deflocculation. These results also elucidated the importance of divalent cations in the maintenance of microbial viability and the integrity of their outer membranes, since their sequestering led to cell lysis like symptoms and the hampering of metabolic activities. The cationic content in the system directly promoted interactions between EPS and floc components through cationic bridging, while also being essential for the normal growth and functioning of the sludge microbial communities. Altogether, the divalent cations have a more considerable impact on the floc ecosystem and can influence the floc maintenance process more acutely than previously reported. Their impact on the microbes and floc ecosystems must be

CHAPTER 7

considered more carefully when establishing the ionic conditioning measures for optimal flocculation of sludge samples.

This is especially important in WWTPs, where the influent ionic composition must be monitored and normalized to ensure a consistent conditioning strategy for the types of sludge flocs required. The addition of cations like calcium and magnesium during chemical flocculation of the sludge samples will greatly influence the short-term and long-term structural and functional stability of the sludge floc ecosystem and this must be considered more seriously. Changes in influent ionic composition must be identified early and rectified to ensure consistency in the ionic conditioning of the sludge flocs resulting in stronger and more stable flocs. The increase in soluble cation composition in the supernatant or bulk solution can be an indicator of issues with the sludge flocs in the system since their incorporation into the sludge floc ecosystem is now well established. Thus, suitable deflocculation mitigation strategies can be developed by monitoring the overall ionic (especially cation) composition in the different fractions of the sludge ecosystem. The effective monitoring of fraction specific ionic composition can be performed by the development of suitable sensors, which are positioned strategically in the treatment system. More work must be considered in this direction since effective maintenance of sludge flocs is more energy and cost effective than developing large scale mitigation strategies.

7.2 Importance of long-term conditioning on the stability of sludge floc ecosystem

The results of this dissertation studies also elucidate the complexities of the biological sludge flocculation process and the need to evaluate it in relative terms instead of in absolute values. Each sludge and its microbial composition will be influenced by the

CHAPTER 7

long-term conditions that they are exposed to and will differ depending on treatment type, influent wastewater content and environmental conditions in the local region. This in turn will have an impact on the quality and quantity of EPS produced, the type of floc stabilizing interactions cultivated and the sensitivity to deflocculative stresses. Short-term transients in conditions are less likely to affect the sludge floc composition and flocculation state in the system unless the microbial communities, the EPS or other floc stabilizing interactions (ionic interactions) were adversely affected. The deflocculative effects of these ‘shock’ events can be reversible in case of transformation of the toxic compound (like the conversion of EDTA to Ca-EDTA) or its removal from the system. Contrastingly, long-term adverse effects and irreversible deflocculation can be brought about by the continued presence of toxins in the system possibly due to their non-biodegradability or due to their interaction with floc bio-components (like the inhibitory and interactive effects of sodium azide). While low DO deflocculation might not be universal, the importance of microbial communities and the cationic content on biological floc maintenance cannot be ignored. Accurate and consistent monitoring of these factors can better indicate the state of the biological flocs in the system while also allowing for the development of better control strategies for the maintenance of strong and well settling flocs.

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