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Identification and Characterisation of Chloramine Decaying Proteins and Control of Impact in Chloraminated Systems

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**This thesis is presented for the degree of
Doctor of Philosophy
of
Western Sydney University**

DEDICATED TO

My beloved son, Sadev

And

Daughter, Sayuri

with my gratitude for their support and tolerance throughout my study

Declaration

This thesis is submitted in fulfilment of the requirements for the Doctor of Philosophy at the Western Sydney University; School of Computing, Engineering and Mathematics. The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not previously submitted this material, either in full or in part, for a degree at this or any other institution. Unless otherwise stated, all of the data and observations presented here are the results of my own work.

Signature: █████ ………………………….

Date: 09/09/2019

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Thesis related publications

Journals

- **Herath, BS**, Torres, A & Sathasivan, A 2018, 'Effects of feed water NOM variation on chloramine demand from chloramine-decaying soluble microbial products during rechloramination', Chemosphere, vol. 212, pp. 744-54. (Chapter 4)

- **Herath, BS** & Sathasivan, A 2019, 'The chloramine stress induces the production of chloramine decaying proteins by microbes in biomass (biofilm)', Chemosphere, p. 124526. (Chapter 6)

Other publications

Book chapter

- Sathasivan, A, **Herath, BS**, Senevirathna, S & Kastl, G 2017, 'Dechlorination in wastewater treatment processes', in Current Developments in Biotechnology and Bioengineering, Elsevier, pp. 359-80.

Conference proceedings

- An oral presentation titled " Effect of feed water DOC on abundance of SMPs in severely nitrified water" presented at the **2015** International Conference on Challenges in Environmental Science & Engineering (Sydney, Australia). (related to Chapter 5)

- A poster titled "Separation of SMPs accelerating chloramine decay in nitrified chloraminated systems" presented at the **2016** World Water Congress & Exhibition (Brisbane, Australia). (related to Chapter 4)

- **Herath, BS** & Sathasivan, A 2016, 'Separation of SMPs Accelerating Chloramine Decay in nitrified chloraminated systems', paper presented to IWA World Water Congress & Exhibition Brisbane, Queensland Australia, 8 - 13 October 2016. (related to Chapter 4)

- A poster titled "Production of chloramine decaying protein as a stress response" presented at the **2016** International Conference on Challenges in Environmental Science & Engineering (Kaohsiung, Taiwan). (related to Chapter 6)

- An oral presentation titled " SMP in chloranminated drinking water systems" presented at the **2017** International Conference on Challenges in Environmental Science & Engineering (Kunming, China). (related to Chapter 8)

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List of Abbreviations

AOA	Ammonia-oxidizing Archaea
AOB	Ammonia oxidising bacteria
AOC	Assimilable organic carbon
BAC	Biological activated carbon
BDOC	Biodegradable organic compounds
BRC	Biostable residual concentration
BSA	Bovine serum albumin
CCA	Canonical correspondence analysis
cCBB	Colloidal coomassie brilliant blue
CDP	Chloramine decaying proteins
C.D.I	Chloramine decay index
CHAPS	[(cholamidopropyl) dimethylammonio]-1-propane sulfonate
cSMP	Chloramine decaying soluble microbial products
DBP	Disinfection by-products
DOC	Dissolved organic carbon
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
2DE	Two-dimensional electrophoresis
EPS	Extra polymeric substances
F_m	Microbial decay factor
GAC	Granular activated carbon
HDPE	High density poly-ethylene
HPC	Heterotrophic plate count
IEF	Isoelectric focussing
IPG	Immobilised pH gradient
k_c	Chemical decay coefficient
kDa	Kilodalton, unit of mass
k_m	Microbial decay coefficient

k_t	Total decay coefficient
LC/MS/MS	Liquid chromatography- multistage mass spectrometry
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NOB	Nitrite oxidising bacteria
NMR	Nuclear magnetic resonance
OTUs	Operational taxonomic units
PCR	Polymerase chain reaction
PD	phylogenetic diversity
QIIME	Quantitative insights into microbial ecology
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMP	Soluble microbial product
TAN	Total ammoniacal nitrogen
TBP	Tributyl phosphine
TCl	Total chlorine
TOC	Total organic carbon
UF	Ultrafiltration
UV	Ultraviolet
WFP	Water filtration plant

Abstract

Chloramine is the second most popular disinfectant behind chlorine used in water distribution systems. The main advantages of using chloramine over chlorine are; it provides a longer lasting disinfectant residual and forms a less amount of the regulated halogenated disinfection by-products. However, at times, microbial chloramine decay can overwhelm stability and is identified as one of the serious problems that needs addressing. One of the mechanisms of microbial chloramine decay is by production of soluble microbial products (SMP), which substantially affects the chloramine decay. The SMP are usually composed of proteins, polysaccharides, humic acids, fulvic acids, nucleic acids, enzymes and structural compounds, but it was suspected that the chloramine decaying SMP could be protein(s) due to the catalytic effect that was noted in the samples. It was noted in soluble form in water after the onset of nitrification in a chloraminated system. When the SMP was filtered out of the water and was run on the SDS page to identify the compound 25 different proteins were seen with weaker signals. Therefore, the identity of chloramine decaying soluble microbial products (SMP), which microbes produce them and how to control them are not known.

If SMP was produced by nitrifiers and since nitrifiers could be inhibited by higher organic carbon levels when changing dissolved organic carbon (DOC) level in the water, it could alter the production of chloramine decaying SMP. Therefore, batch rechloramination tests were conducted for reactor sets with variable DOC levels to understand their effect on the impact of SMP. The results revealed as the highest production of SMP had been stimulated with low DOC level (0-1mg-C.L^{-1}) compared to other DOC levels (2-3, 4-5 and 7-8 mg-C.L^{-1}). To recognize microbial community variations with the impression of identifying CDP producing micro-organisms, microbial community analysis was also carried out in the same reactor sets. Significant differences in bacterial types against DOC variations could not be detected. However, some of the bacterial types such as *Micobacterium*, AOB, *Bradyrhizobium sp.*, *Methylobacterium* and family Sphingomonadaceae recognized in this study are known to produce extracellular polymeric substances (EPS).

My early work has identified that SMP are proteins; hence, named as chloramine decaying proteins (CDP). In Relation to the context of these experiments, the proteins consisting within EPS can be considered as CDP. Questioning if nitrifiers always produce CDP, two nitrified reactors - one with chloramine (chloraminated reactor) and the other with ammonia (ammoniated reactor) - were operated using nutrient added Milli-Q water as the feed water in

a way nitrification occurs within the reactor. MilliQ was selected since it produced the highest concentration of CDP. It was expected that CDP could be easily separated amongst 25 previously found proteins. Therefore, nitrified bulk water and biofilm samples from both reactors were subjected to protein separation (2-dimensional gel electrophoresis and SDS-polyacrylamide gel electrophoresis) and protein identification (mass spectrometry-MS). Furthermore, bacterial community variations on ammoniated and chloraminated reactors were characterised by sequencing of 16S RNA.

The batch rechloramination results obtained from the reactors for the first time established the production of CDP as a microbial response to chloramine stress. The bacterial community characterisations on each of the reactors did not show major differences in identified bacterial strains. However, the EPS producing bacterial strains (AOB, *Bradyrhizobium sp*. and family Sphingomonadaceae) identified in chloraminated reactors were suspected to be responsible for CDP production. Chloraminated and ammoniated bulk water samples were not resulting in enough concentrations, therefore, for comparison of protein spots and MS analysis, the biofilm samples (which are believed to have more CDP) were analysed. The major proteins detected were ammonia monooxygenase subunit A and putative porin related to *Nitrosomonas sp.* and *Bradyrhizobium sp.*, respectively. However, their relation to CDP has to be further investigated.

Conclusively, every aspect of this study is directing towards discovering a better control mechanism for the microbial/ CDP induced accelerated chloramine decay. Silver is a known inhibitor for several micro-organisms. Therefore, experiments were conducted to reveal the optimum dose of silver on inactivating nitrifying microbes and CDP for controlling the fast decay of chloramine. Interestingly, 2 μg-Ag.L^{-1} silver (which is far lower than the recommended level- 0.1 mg-Ag.L^{-1}) was found to be effective for improving chloramine residuals in tested bulk waters. This study concludes by further emphasising the need for extensive study/research in further identification of CDP and bacteria communities responsible for chloramine decay in chloraminated drinking water distribution systems.

Chapter 1

Introduction

1.1 Background

Chloramine is the second most popular secondary disinfectant used in many countries for over 90 years due to its stability and formation less regulated disinfection by-products. However, at times maintaining a recommended level of chloramine in the water distribution system is a challenge due to its fast decay followed by nitrification.

Several researches have been undertaken to understand the role of nitrification; which was conventionally considered as the major cause of diminishing chloramine (Sathasivan, Fisher & Tam 2008; Skadsen 1993). Nitrifiers are responsible for the nitrification process, in which ammonia is converted to nitrite by ammonia oxidising bacteria (AOB), followed by conversion of nitrite into nitrate by nitrite oxidising bacteria (NOB).

Questioning the traditional belief, Sathasivan et al., 2005 developed the microbial decay factor method to measure microbial and chemical chloramine decay in chloraminated samples. They noted substantial (about two to three times chemical decay) microbial chloramine decay occurring even before the onset of nitrification. Based on further examination of samples obtained from chloraminated water supply systems, they noted even faster (about five times the total decay before the onset of nitrification) acceleration upon the onset of nitrification. This phase was named as severe nitrification. Upon further experimentation, soluble microbial

products (SMP) in severely nitrifying water was found to be responsible for the acceleration (Krishna, Sathasivan and Sarker (2012).

Laspidou and Rittmann (2002) explained that extra cellular polymeric substances (EPS) produced by bacterial biofilm act as a protective barrier that provides resistance to harmful effects. EPS matrix protects bacteria from external stresses (Lewandowski & Beyenal 2013) in various ways such as chemically reacting with and neutralizing the antimicrobial agent (Dodds, Grobe & Stewart 2000). The soluble component of EPS are considered as SMP (Laspidou & Rittmann 2002) and are closely associated with EPS that is bound to bacterial cells (Janga et al. 2007). EPS is generally composed of proteins, carbohydrates, nucleic acids, lipids and humic substances (Neu, Flemming & Wingender 1999). However, Majority of EPS consists of proteins and carbohydrates (Liu, Yan & Fang 2003; Neu, Flemming & Wingender 1999). Considering that chloramine is harmful (an antibacterial agent) for bacteria living in chloraminated systems, chloramine decaying soluble microbial products could be a part of EPS.

Chloramine decaying microbial products which is identified as SMP catalyses the auto-decomposition and nitrite oxidation resulting in much higher chloramine decay when compared against nitrifying bacterial chloramine decay (Krishna, Sathasivan & Sarker 2012). Assuming this SMP as proteins (proteins are known to have catalytic effect (Cooper 2000)), I have conducted several experiments aiming to identify this soluble products decaying chloramine. My previous work showed that the chloramine decaying proteins (CDP) in the severely nitrified bulk water samples could be separated using 30 kDa and 50 kDa cut off membrane filters. There were more than 20 proteins in the range 30-50 kDa, in which some are identified as common drinking water bacterial types (e.g. *Bradyrhizobium sp., Nitrosomonas sp., Acidovorax sp.,* Family Mycobacteriaceae *and Hyphomicrobium sp.*). With the intention of controlling fast decay of chloramine, it is necessary to identify the specific chloramine accelerating protein(s) and/or the microbial strains which produce these proteins in chloraminated water distribution systems.

However, Sarker (2012) reported that there can be situations where CDP type behaviour is not noted under nitrifying conditions. Furthermore, chloramine decay promoted by both autotrophs (nitrifiers) and heterotrophs were newly reported (Herath, Sathasivan & Lam 2015). However, the impact of CDP could not be identified in samples from reactors fed with higher DOC concentrations in this study. Furthermore, temperature, pH, the presence of silver and water

retention time could also affect the presence/production of CDP. These parameters are usually fluctuating in chloraminated distribution systems and variations may host different microbial communities being responsible for the behavioural changes of CDP. Therefore, a systematic investigation into the effect of these factors on CDP production is essential.

1.2 Research objectives

Controlling of chloramine decay in drinking water distribution systems is a necessity as it consumes substantial resources annually. There are numerous vital factors such as CDP, nitrifiers, heterotrophic bacteria which mandates attention for further investigation into this type of induced chloramine decay. CDP have been observed under severe nitrification conditions, but organic matter could also play a role in controlling the nitrification and thus have an impact on CDP. Therefore, this study investigates the effect of natural organic matter (NOM) variations on different nitrifying conditions and the related bacterial community followed by impact of NOM on CDP. In the past, there were several researches that successfully investigated the production of EPS as a resistance mechanism against harmful effects and/or external stress. Therefore, the production of chloramine decaying compounds was investigated with reactors being maintained under specific conditions. The most significant aspect of the research, the identification of CDP, was implemented using CDP rich water samples. Furthermore, attributes of silver inhibition on nitrifying organisms and CDP were explored for the purposes of controlling chloramine decay. The research objectives were as follows:

- To recognize the microbial community variations in different DOC levels along with the identification of CDP focusing on chemical decay.

- To investigate the CDP production mechanism by providing favourable conditions such as low DOC and chloramine stress to induce the CDP impact in nitrified water.

- To identify the protein(s) and micro-organism(s) which are specifically responsible for accelerated chloramine decay.

- To identify the effect of silver on nitrifying micro-organisms (on severely nitrified conditions and on CDP for the better control of accelerated chloramine decay.

1.3 Significance of the research

Chloramine is widely used over 90 years as a safe and effective disinfectant by municipally run and privately owned water systems across the United States and Canada. Great Britain is one among few European countries which uses chloramines for residual disinfection in the distribution network for the reduction of disinfection by-products. Finland, Spain and Sweden also occasionally use chloramines for disinfection. Sudden disappearance of chloramine followed by the onset of nitrification in the distribution system is the biggest problem, and yet a problem to be solved despite the majority of research implemented within the last four to five decades. Accelerated chloramine decay in water distribution systems is the result of both nitrification and catalytic reaction of CDP with chloramine. As these chloramine decaying soluble microbial products could not be identified consistently and during all the time in nitrified chloraminated systems, it could be assumed that there could be some limiting factors affecting their production. Therefore, this research was conducted, with the aim of controlling accelerated chloramine decay. The consequences of the research outcomes are:

- This research reported that the CDP are produced as a stress response to chloramine. One of the major difficulties witnessed in identifying this CDP was the low concentration of CDP. Hence, applying chloramine stress, CDP was produced in sufficient concentrations for the identification.

- Nitrified conditions are induced with the presence of free ammonia. In chloraminated water, free ammonia is made available with chloramine dosing and chloramine decay. Therefore, identifying CDP and microbial community diversities in nitrified conditions induced by both environments, one being with only ammonia and the other being with only chloramine would help in narrowing down the proteins produced/released due to chloraminated conditions. This innovative finding facilitates the application of better control mechanisms for CDP induced chloramine decay.

- Diversity of microbial communities under different DOC levels with respect to various water quality parameters (DOC, total chlorine, ammonia, nitrite) was investigated. This helps to manage the chloramine residual level under different seasonal variations as DOC levels could vary even in the same location.

- Different concentrations of silver were applied as an inhibitor on various nitrified conditions (mild nitrification and severe nitrification) and on CDP. This will be useful

as a chloramine decaying inhibitor for Sydney Water and all other water supply systems in the world which use chloramine as their disinfectant.

1.4 Research approach

With the aim of fulfilling research objectives under laboratory conditions and due to the difficulties of obtaining consistent and expected conditions in a full-scale water distribution system, the lab scale reactor systems and reactors were operated in the Western Sydney University, Kingswood for the purposes of this study. Feed water was regularly collected from the Orchard Hills water treatment plant and reactor sets were operated with different DOC levels, depending on seasonal differences and induced conditions such as treated with biologically activated carbon treated water. Each reactor set consisted of 5 reactors connected in series were operated under controlled conditions (initial chloramine residual, pH and temperature). Separately, 2 reactors; namely Chloraminated (with chloramine only) and Ammoniated (with ammonia only) were maintained using with nutrients added ultra-pure water, which were initiated with identical nitrified seed water samples collected from one of the above explained reactor sets. The reactors and reactor sets facilitated the investigation of unknown factors behind the accelerated chloramine decay in more details (e.g. identification of CDP, suspected bacterial types, cause of CDP production and CDP control methods).

This study comprises of ten chapters. Chapters 4 to 9 are presented as individual chapters which have already been published, submitted to journals or prepared for publication as peer-reviewed journal publications. However, to facilitate easy reading, common materials and methods are detailed in Chapter 3. The experimental design and the outcomes of this research have been detailed in Figure 1.1. Chapters 4, 5 and 9 were written on experiments conducted utilising the lab scale reactor systems consisting of variable DOC concentrations. Chapters 6 to 8 state the core experiments directed with ammoniated and chloraminated reactors based on the identification of CDP and the microbial diversity related to CDP production.

- Chapter 1 introduces the thesis including research objectives, their significance and the approach employed in this dissertation.

- Chapter 2 reviews the literature, which includes prevailing research efforts implemented to understand chloramine decay and strategies (corrective and preventative) that have been deployed for maintaining an adequate chloramine residual

in the system. With the direction of identifying chloramine decaying microbial products, protein separation and identification techniques are also embedded in to the literature review section. Furthermore, diversity of microbial communities and their characteristics were also introduced in this chapter.

- Chapter 3 organizes the common materials and methods employed in this research, which include the sampling methods, operation and maintenance of lab scale reactors and reactor sets and chemical analytical procedures.

- Chapter 4 reveals the effects of feed water NOM variation (as a measurement of DOC) on chloramine demand from CDP during re-chloramination by exploring the physicochemical parameters (total chloramine, total ammoniacal nitrogen, nitrite, nitrite, pH, etc)

- Diversity and similarity of microbial communities characterised by sequencing of 16S RNA under various nitrification conditions on same reactor sets fed with waters containing different DOC levels as described in Chapter 4 are presented in Chapter 5.

- Chapter 6 identifies the production of CDP as a response to stress by investigating physiochemical parameters after batch re-chloramination tests were carried out on reactors separately maintained with chloramine (Chloraminated reactor) and ammonia (Ammoniated reactor).

- Chapter 7 presents the diversity and similarity of the microbial communities characterised by sequencing of 16S RNA in chloraminated and ammoniated reactors described in chapter 6. This helps in understanding the differences between CDP producing and non-CDP producing microbial strains.

- CDP separation and identification techniques are described in Chapter 8. This facilitates the narrowing down of identified proteins from my previous study based on CDP production as a key finding in this study.

- Chapter 9 deals with the impact `optimum silver dose on inactivating nitrifying microbes, heterotrophic microbes and CDP by batch re-chloramination.

- Chapter 10 reports the conclusions of this study and recommendations for future study.
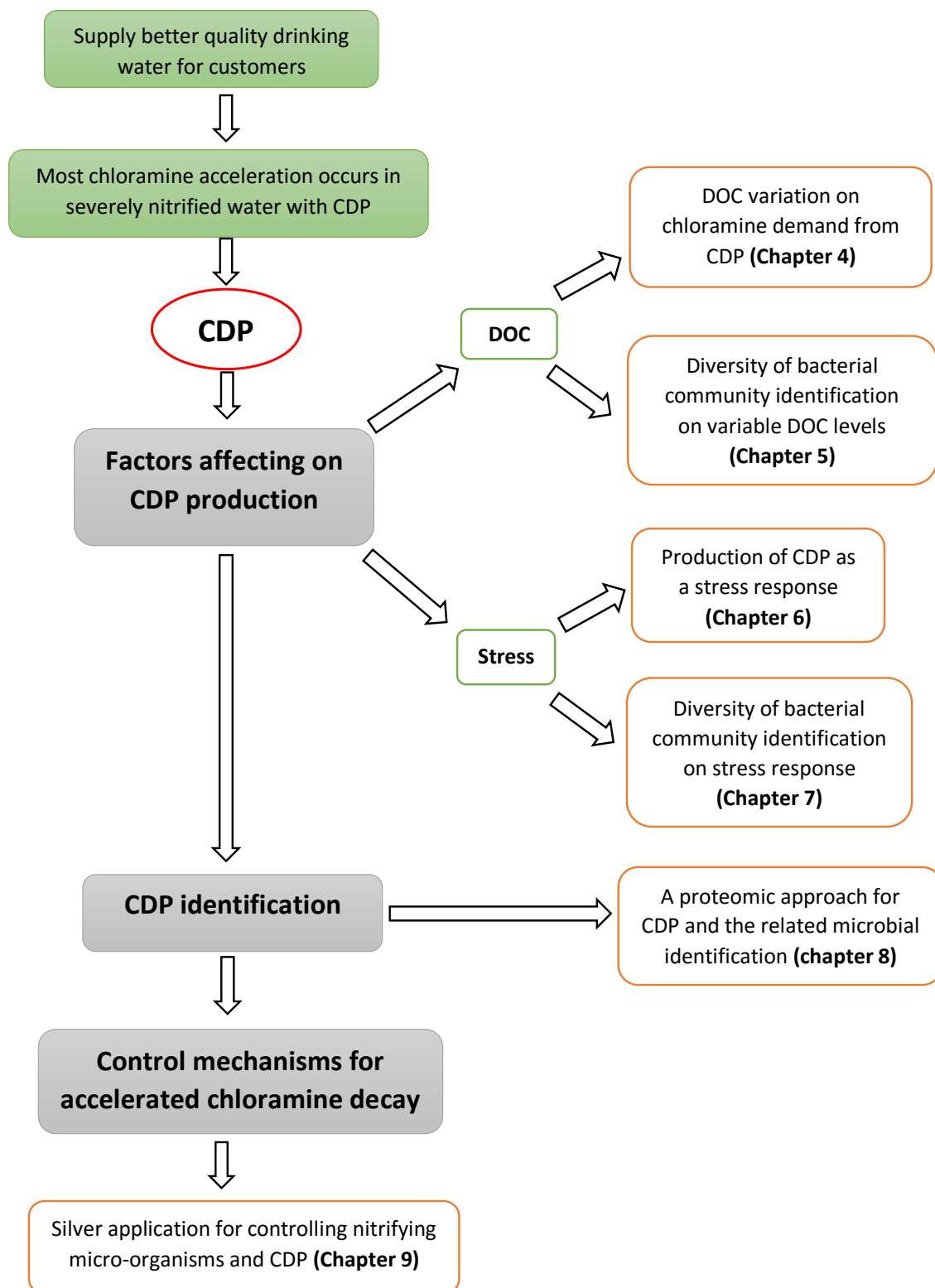
Figure 1.1: The experimental design and the outcomes of this research

Chapter 2

Literature review

2.1 Drinking water disinfection

Tap water is disinfecting to shield public health from disease causing microorganisms. Disinfectants kill bacteria, viruses and other organisms that cause deaths and serious illnesses such as typhoid, hepatitis and cholera in water. Most commonly used disinfectant to treat drinking water is chlorine. Other disinfectants are chloramine, ozone, chlorine dioxide, and ultraviolet (UV) light. While water moves through the pipelines to the consumers, certain amount of disinfectant has to be maintained. The disinfectant also needs to comply with other regulatory requirements and should be cost effective. Therefore, chloramine and chlorine is frequently used in distribution systems. The drinking water standard for chloramines is 4 parts per million (ppm) measured as an annual average.

2.2 Chloramine

Chloramine becomes an attractive secondary disinfection choice over chlorine due to many advantages. Mainly; chlorine gives water a taste, form cancer-causing compounds (which is called disinfection by-products-DBP) by combining with organic contaminants and reduce the effectiveness with the presence of turbidity (cloudy water) (Okpara, Oparaku & Ibeto 2011). However, chloramine offers many advantages such as, improvement of taste and odor, lower

concentration of chlorinated DBP formation and ability to reduce total coliform and heterotrophic bacteria (Neden et al. 1992; Norton & LeChevallier 1997). Importantly, providing longer lasting residuals throughout water distribution systems made chloramine applicable for long water distribution systems or systems having a long water retention time.

Sydney Water, Australia started to use chlorine as a disinfectant in the water distribution system since 1940s. Since, the early 1960s the first trial of chloramination commenced in some distribution systems of Sydney Water. Currently, Sydney Water has seven chloraminated and six chlorinated water systems. Beside Sydney Water, Goldfield and Agricultural Water Supply System, Western Australia, water utilities located in country side of South Australia and most water utilities in Queensland use chloramine as a secondary disinfectant. Still, there is no convincing record of how many water utilities have been using chloramine as a secondary disinfection in Australia. However, it is known that most water utilities in Australia that have long water retention times or long distribution systems are using chloramine as a secondary disinfectant. Chlorination has been practiced in short or small water distribution systems.

Chloramine is formed by mixing chlorine with ammonia. There are different types of chloramines. They are monochloramine, dichloramine, trichloramine and organic chloramines. Monochloramine is the most common form used as a secondary disinfectant in drinking water systems. Dichloramine, trichloramine, and organic chloramines are formed in lower levels in chloramine application (White 2010).

The pH, the ratio of chlorine to ammonia-nitrogen and to a lesser extent, temperature and contact time controls the production of monochloramine, dichloramine and trichloramine and hence the decay rate of chloramines. The generalized formation reactions of these three forms of chloramine are shown in the following Eq. 2.1 to 2.3 (Haas 2000). A pH between approximately 7.5 and 9.0 is optimum for the formation of monochloramine and ideal pH is 8.3 (Eq. 2.1) (Hammerl & Klapötke 2006). Dichloramine is formed when the chlorine to ammonia-nitrogen weight ratio is greater than 5:1; however, this reaction is very slow (Eq. 2.2). Organic chloramines are formed when chlorine reacts with organic nitrogen compounds (Eq. 2.4). Trichloramine formation does not usually occur under normal drinking water treatment conditions (Eq. 2.3) (Hammerl & Klapötke 2006).

$$HOCl + NH_3 \longrightarrow NH_2Cl \text{ (monochloramine)} + H_2O \qquad \text{(Eq. 2.1)}$$

$$HOCl + NH_2Cl \longrightarrow NHCl_2 \text{ (dichloramine)} + H_2O \qquad \text{(Eq. 2.2)}$$

$HOCl + NHCl_2 \longrightarrow NCl_3$ (trichloramine) $+ H_2O$ (Eq. 2.3)

$HOCl + $ Organic nitrogen $\longrightarrow R\text{-}NHCl$ (organic chloramine) (Eq. 2.4)
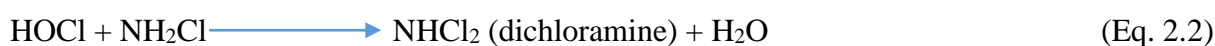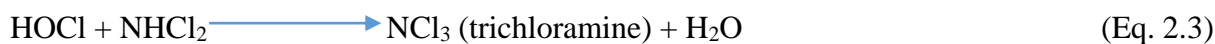
2.3 Possible chloramine decay mechanisms in water distribution systems

After the formation of chloramine, it starts to decay due to chemical and biological reasons. The decay rate is normally controlled by water quality including chlorine to ammonia-N ratio, temperature, pH, carbonate, organic matters, bromide, nitrite, atmospheric exposure, iron etc.(Vikesland, Ozekin & Valentine 2001; Vikesland & Valentine 2000; Vikesland, Valentine & Ozekin 1996). As a result of chemical decay of chloramine, free ammonia is released to water which boosts nitrification process in the chloraminated distribution system. Major chloramine decay pathways are described below.

2.3.1 Auto decomposition

Chloramine decay occurs even without reducing agents called auto decomposition. In auto decomposition, chloramine undergoes in the distribution system by a complex set of reactions that eventually oxidized ammonia and reduce the quantity of active chlorine in the system (Vikesland, Ozekin & Valentine 2001). A simplified form of stoichiometry is presented in Eq. 2.5 (Vikesland, Valentine & Ozekin 1996).

$3NH_2Cl \longrightarrow N_2 + NH_3 + 3Cl^- + 3H^+$ (Eq. 2.5)
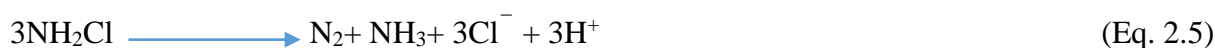
This is highly a pH dependant reaction. Low pH levels promote the auto decomposition than in higher pH levels. Therefore, maintaining a lower pH level affects negatively on chloraminated systems. Apart from the pH, temperature also plays a major role on auto decomposition. Increasing temperature by 17 °C can double the chloramine decay rate. Additionally, Valentine, RL (1998) reported the amplification of decay rate by 6.5 times when the temperature increased 4 °C to 35 °C at pH 7.5. However, there are many other factors relate to the decay of chloramine than temperature. Inorganic/organic carbon residuals, chlorine to ammonia-N ratio, and initial chloramine concentrations are other major parameters that could affect the auto-decomposition process (Vikesland, Ozekin & Valentine 1998; Vikesland & Valentine 2000).

At or over pH 8.3, chlorine-to-ammonia-N ratio is less effective on accelerating chloramine decay. However, to control the chloramine decay, chlorine-to-ammonia-N weight ratio should

be approximately 3:1 (Vikesland, Ozekin & Valentine 1998). In distribution systems, free ammonia residuals can be limited by maintaining chlorine-to-ammonia-N ratio between 4.5:1 and 4.7:1 (Kirmeyer 2004). Experiments done by Vikesland, Ozekin and Valentine (1998) indicated that the role of natural organic matter (NOM) as a catalyst is negligible and the reactions with NOM occur due to the redox and substitution reactions with monochloramine. Moreover, direct interaction between NH_2Cl and NOM matrix could result DBPs formation in chloraminated systems. However, bromide ions act as a catalyst in accelerating the auto-decomposition reaction by producing nitrogen gas and free ammonia (Vikesland, Ozekin & Valentine 2001).

In water distribution systems, chloramine auto-decomposes by air and sunlight when it is exposed to the atmosphere and the decay rate is controlled by temperature, sunlight intensity and wind velocity (Kirmeyer 2004).

2.3.2 Nitrite oxidation by chloramine

In chloraminated distribution systems, nitrite is mainly produced via nitrification process. In addition, raw water may also contain certain amount of nitrite. It has been shown that the direct reaction between chloramine and nitrite is more favourable than other chemical reactions in normal water conditions (Margerum et al. 1994; Valentine, R 1985) Nitrite oxidation is controlled by pH, temperature, free ammonia, nitrite and initial chloramine residuals. Eq. 2.6 and 2.7 represent the nitrite oxidation which leads to nitrate production and free ammonia (Vikesland, Valentine & Ozekin 1996). Free ammonia is the major component which leads to nitrification in chloraminated water distribution systems.

$$NH_2Cl + NO_2^- + H_2O \longrightarrow NH_4^+ + NO_2^- + Cl^- + NO_3^- \qquad \text{(Eq. 2.6)}$$

$$HOCl + NO_2^- \longrightarrow NO_3^- + H^+ + Cl^- \qquad \text{(Eq. 2.7)}$$
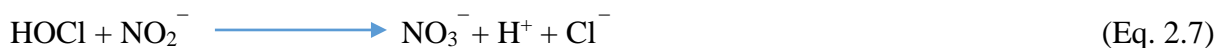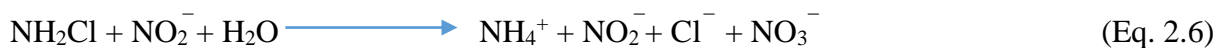
2.3.3 Nitrification

Nitrification is a two-step microbiological process where ammonia is oxidized to nitrite (NO_2^-) by AOB and nitrite is nitrate (NO_3^-) by NOB. A simplified equation for the production of nitrite by ammonia and production of nitrate by nitrite is illustrated in Eq. 2.8 and 2.9, respectively.

Application of chloramine to the distribution system eventually introduces free ammonia in the water. Excess ammonia from chloramine formation and free ammonia release from chloramine decay leads nitrification. The existing free ammonia in the distribution system serves as an energy source for nitrifying bacteria which result in nitrification during the decay process.

$$2NH_4^+ + 3\,O_2 \longrightarrow 2\,NO_2^- + 2\,H_2O + 4\,H^+ \qquad\qquad\qquad (Eq.\ 2.8)$$

$$2\,NO_2^- + 1\,O_2 \longrightarrow 2\,NO_3^- \qquad\qquad\qquad\qquad\qquad (Eq.\ 2.9)$$
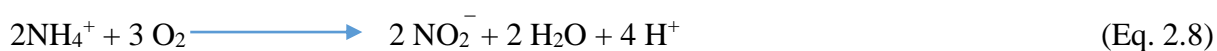
In the United States, it was reported that 63% of water utilities which use chloramine have been suffered from nitrification problems (Wilczak et al. 1996). In South Australia, 64% of the tested samples illustrate nitrification episodes in chloraminated water distribution systems (Cunliffe 1991). Nitrification of chloraminated drinking water deteriorates water quality, including a decrease in chloramine residuals which could lead to increase in heterotrophic plate count (HPC) bacteria and increase in nitrite and nitrate-nitrogen, all of them lead to regulatory violations.

Once nitrification has set in, it is almost impossible to stop it even introducing a high chloramine levels like 6.0 to 8.0 mg.L^{-1} (Odell et al. 1996). In chloraminated water distribution systems total chloramine, free ammonia, temperature, pH, light, dissolved oxygen (DO), water chemistry and microbial community composition controls the nitrification process. When nitrifiers are exposed to minimal concentrations of cyanides, halogenated compounds, phenols, mercaptans, thiourea and metals (iron, aluminium, copper, zinc, manganese, lead, and nickel); they display severe stress (Ford 1981).

After the onset of nitrification, both nitrifying bacteria and their products exert chloramine demand, decreasing the chloramine residual concentration in the distribution system. The presence of nitrifiers and their activity is detected by increased in NOx (nitrite + nitrate) level. This is because nitrite can be oxidized by chloramine or NOB to nitrate in a chloraminated environment, but there is only one mechanism to produce nitrite, which is by AOB. Sathasivan, Fisher and Tam (2008) have observed two distinct stages based on the chloramine decay rate and nitrite production in a chloraminated distribution system as mild nitrification and severe nitrification. In mild nitrification, chloramine decay is reasonably stable and nitrite level normally remained less than 0.010 mg-N.L^{-1}, while in severe nitrification, chloramine decay accelerates excessively and reaching a nitrite level of more than 0.1 mg-N.L^{-1}. Severe nitrification triggers when the chloramine level reached below 0.5 mg.L^{-1} (Sathasivan, Fisher & Tam 2008).

However, both chemical and microbial decay occurs in a full-scale distribution system. In order to understand the decay mechanism, separating microbes (including nitrifying bacteria) and chemical reactions is required. Sathasivan, Fisher and Kastl (2005) proposed a microbial decay factor (F_m) method by summarising the problems of traditional indicators of onset of nitrification such as nitrite, nitrate, pH, ammonia and chloramine residuals. It is a simple tool which is the ratio between microbial and chemical decay coefficients, that was established for separating and quantifying the microbial activities and chemical reactions on chloramine decay (Sathasivan, Fisher & Kastl 2005). The chemical decay coefficient (k_c) is affected by chloramine residual, total chlorine to ammonia ratio, temperature, total organic carbon, pH, nitrite and other agents present in water; while the microbial decay coefficient (k_m) depends on activities of microorganisms. Chloramine stability and an early warning of the onset of nitrification can be precisely assessed by this method.

2.3.4 Microbial products

Soluble microbial products (SMP)

Soluble cellular components which are released by microorganisms called Soluble Microbial Products (SMP). It can be released during microbial cell lysis or cell synthesis and normally diffuses through the cell membrane. Furthermore, SMP can be released for other purposes as well. They consist of a wide range of high (>50 kDa) to low molecular weight (<0.5 kDa) compounds that include proteins, polysaccharides, humic acids, fulvic acids, nucleic acids, enzymes and structural compounds (Parkin & McCarty 1981; Rittmann, Bruce Edward et al. 1987).

Few studies have been conducted to investigate SMP in drinking water systems except for a very few literatures on its formation process (Carlson & Amy 2000). Since the demand of high quality drinking water has increased worldwide, SMP in drinking water distribution system should be further studied (Jarusutthirak & Amy 2007). Many studies reported that nitrifying bacterial activities (especially AOB) release SMP (Noguera et al. 2009; Regan, John M, Harrington & Noguera 2002; Wolfe et al. 1990) that could deplete the chloramine residual by exerting demand on chloraminated system. Furthermore, if this produced SMP stimulate heterotrophic bacterial regrowth, it will be able to detect using heterotrophic plate count

(Allen, Edberg & Reasoner 2004; Bartram, Jamie et al. 2003; Bartram, J et al. 2004; Sartory 2004).

Krishna, Sathasivan and Sarker (2012) recently demonstrated that chloramine decaying substances which is released or produced in the nitrified chloraminated systems accelerates chloramine decay. They also reported that; applying protein denaturing techniques such as silver addition (presented in Fig. 2.1), subjecting to high temperature and high pH completely terminated the accelerated chloramine loss. This verified the SMP could be a protein-related compound; however, further confirmation is essential. Repeated re-chlorination in filtered severely nitrified waters demonstrated that the SMP act like catalyst to boost the auto-decomposition and nitrite oxidation on chloramine decay. However, the identity of SMP (what exactly this compound is), source of the compound and the decay mechanism of chloramine under severely nitrifying conditions still to be investigated.
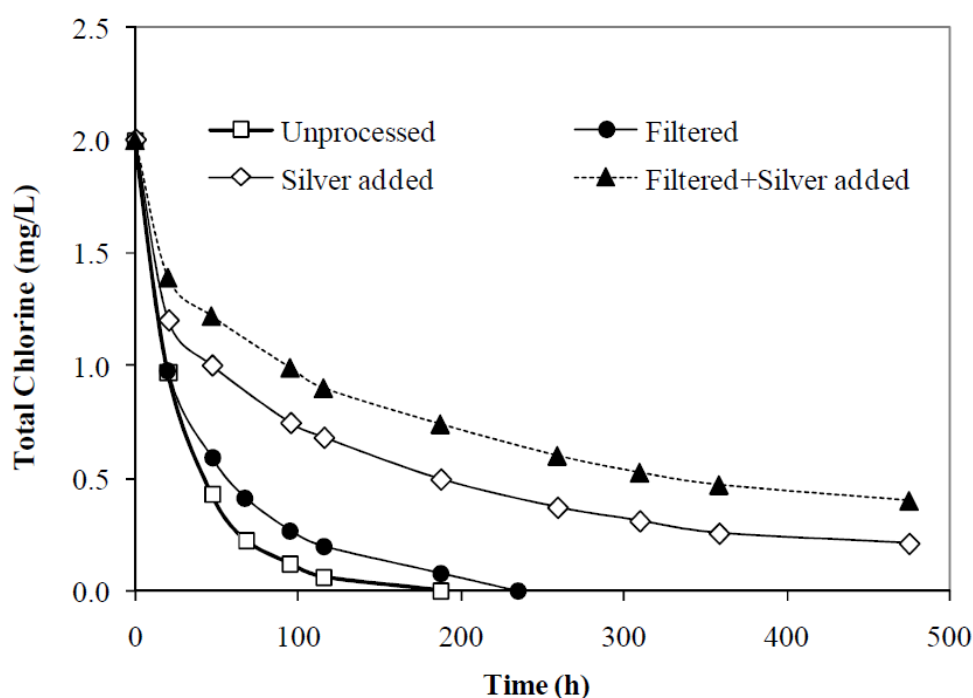
Figure 2.1: Total chlorine decay profiles in severely nitrified water samples after addition of silver and without addition. 'Unprocessed' represents the not filtered samples where 'filtered' represents the filtered samples through 0.22 μm.

(Adapted from Bal Krishna et al. 2012)

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Extra cellular polymeric substances (EPS)

Due to the increased susceptibility to disinfection in planktonic phase (Simões, M et al. 2003; Simões, M, Pereira & Vieira 2005), most of the microorganisms are grow in aggregated forms such as biofilms and flocs. In most biofilms major percentage (90%) is accountable for extra cellular polymeric substances (EPS) (Flemming & Wingender 2010) which mostly produced by bacteria and in which the biofilm cells are embedded. Laspidou and Rittmann (2002) describes number of functions of the EPS such as aggregation of bacterial cells in flocs and biofilms, adhesion to surfaces, stabilization of the biofilm structure and formation of protective barrier that provides resistance to harmful effects. Other than those functions EPS accumulate the nutrients from the environment via sorption of exogenous organic compounds, accomplish enzymatic activities such as digestion of exogenous macromolecules for nutrient acquisition.

EPS are divided into the soluble components known as soluble EPS or SMP and into the extracellular components that are attached to the biomass known as bound EPS. The composition of EPS and SMP could be almost similar or identical as soluble EPS (SMP) are dissolution of bound EPS (Nuengjamnong et al. 2005).

The major components of EPS are polysaccharides, proteins and DNA (Flemming & Wingender 2010). Earlier it was believed that the majority of the matrix are accountable for polysaccharides. However, protein and nucleic acids as well as amphiphilic compounds including phospholipids have also been shown to appear in significant amount or even predominate (Flemming & Wingender 2010; Wingender, Neu & Flemming 1999, 2012). As described earlier, EPS act as a protective barrier that provides resistance to harmful effects and consists of protein. Therefore, there is a high possibility this chloramine decaying agent could be a component/s of EPS. Nevertheless, it has to be confirmed.

In chloraminated water distribution systems, the presence of both autotrophs (nitrifiers) and heterotrophs were identified. Furthermore, nitrifiers produce EPS or/and SMP (Jiang et al. 2008; Rittmann, Bruce E, Regan & Stahl 1994; Sepehri & Sarrafzadeh 2018) in certain scenarios. Other than nitrifiers, heterotrophic bacteria such as *Mycobacteria* (Chapman 2003) and *Methylobacterium* (Tsagkari & Sloan 2018) produce EPS in drinking water systems. Therefore, the impact of SMP/accelerated chloramine decay can be accountable for the nitrifies or heterotrophs living in nitrified chloraminated condition.

Several functions and the EPS component involved can be identified as presented in the Table 2.1 adopted from Flemming and Wingender (2010).

Table 2.1: Functions of extracellular polymeric substances (EPS) in bacterial biofilms

Function	Relevance for Biofilms	EPS components involved
Adhesion	Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces	Polysaccharides, proteins, DNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition	Polysaccharides, proteins and DNA
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with proteins multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication	Neutral and charged polysaccharides, (such as amyloids and lectins), and DNA
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments	Hydrophilic polysaccharides and, possibly, proteins
Protective barrier	Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protozoa	Polysaccharides and proteins
Sorption of organic compounds	Allows the accumulation of nutrients from the environment and compounds the sorption of xenobiotics (thus contributing to environmental and detoxification)	Charged or hydrophobic polysaccharides and proteins
Sorption of inorganic ions	Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (contributing to environmental detoxification)	Charged polysaccharides and proteins, thus including inorganic substituents such as phosphate and sulphate
Enzymatic activity	Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms	Proteins
Nutrient source	Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community	Potentially all EPS components
Exchange of genetic information	Facilitates horizontal gene transfer between biofilm cells	DNA
Electron donor or acceptor	Permits redox activity in the biofilm matrix	Proteins (for example, those forming pili and nanowires) and, possibly, humic substances
Export of cell components	Releases cellular material as a result of metabolic turnover	Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids
Sink for excess energy	Stores excess carbon under unbalanced carbon to nitrogen ratios	Polysaccharides
Binding of enzymes	Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides	Polysaccharides and enzymes

2.4 Factors impacting on chloramine decay caused by nitrification

As accelerated chloramine decay in distribution systems mostly detected in nitrified water, it is vital to examine the triggering factors for nitrification to apply control measures for chloramine decay. There are a number of factors that can directly influence the nitrification event in the distribution systems include: chloramine residual, total chlorine to ammonia-N ratio, ammonia concentration, retention time, characteristics of distribution system hydraulics and pipelines, pH, NOM and temperature.

The presence of free ammonia in drinking water system produced by excess ammonia from chloramine formation, released by chloramine decay and formed by reactions between nitrate and metals in the system (Zhang, Yan, Love & Edwards 2009). The free ammonia in the system provides the favourable condition for nitrification by the existing nitrifying bacteria (AOB & NOB). Therefore, excess free ammonia present in the system at low chlorine to ammonia ratio tend to encourage nitrification and is one of the primary reasons for high nitrification rate and accelerated chloramine decay (Karim & LeChevallier 2006; Zhang, Yan, Love & Edwards 2009).

The retention time and pipe materials of the distribution system also proposed to play a role in nitrification episode. Long retention time and low chloramine residual in water promote the growth of nitrifying bacteria, thus urging nitrification to govern the system. In addition, certain type of pipe materials (e.g. iron) used in the distribution system may provide favourable environment (stimulate the growth of nitrifying bacteria) for triggering nitrification in the distribution system (Zhang, Yan & Edwards 2009; Zhang, Yan, Love & Edwards 2009).

The pH affect nitrification not only by affecting the growth of nitrifying bacteria, but also by affecting the ammonia released from chloramine decay and chloramine inactivation rate on nitrifying bacteria (Harrington et al. 2002; Oldenburg et al. 2002). The pH decrease during the nitrification episode. At low pH, more free ammonia is released and thus increases the chloramine decay rate.

Low flow and dead ends in the distribution system network provide favourable conditions for accumulating materials to form sediment. Sediment in distribution pipes also exert chloramine demand and further enable nitrifying bacterial growth (Odell et al. 1996; Zhang, Yan, Love & Edwards 2009). They may also create micro-anaerobic environments where oxygen could be limiting. Even though nitrifying bacteria are obligate aerobes, they can use nitrite and nitrate

as an alternative electron acceptor in low oxygen circumstances (Alleman & Preston 1991). Moreover, sediment provides a surface area for bacteria attachment can enhance the resistance of bacteria including nitrifying bacteria to disinfectant (Zhang, Yan, Love & Edwards 2009).

Atmospheric exposure facilitates chloramine degradation due to the sunlight at varying rates depending on the intensity of light (Alleman, Keramida & Pantea-Kiser 1987). Rand and Gagnon (2008) reported the deterioration of disinfectants such as free chlorine and monochloramine when samples are exposed to UV light.

2.4.1 Effect of temperature on nitrification

Usually, most water distribution systems are subject to temperature variations between 6 and 35 °C. Nitrification is highly influenced by water temperature in drinking water distribution systems. The optimal temperature for nitrification and nitrifying bacteria growth is 25 °C to 30 °C (Fisher et al. 2009; Odell et al. 1996). Therefore, nitrification occurrence was higher during summer. Higher temperature also increase the rate of chloramine decay and therefore provides more free ammonia for nitrifying bacterial growth (Fisher et al. 2009; Nowlin 2001). Hence, reduction of the temperature could slow down the chloramine decay and then the nitrification which is responsible for further decaying of chloramine. On the other hand, elevated temperature increase the disinfectant efficiency of chloramine, which could help to control nitrifying bacteria in some cases (Lieu, Wolfe & Means 1993). Finding a balance is a difficult one, but temperature is difficult to control in distribution systems.

According to the biostability concept (Woolschlager et al. 2001) the bacterial regrowth can be prevented if the inactivation rate equals or exceeds the bacterial growth rate at each location within the distribution system. Biostable residual concentration (BRC) is the chloramine residual below which potential of nitrification occurrence exists. The equation for BRC was implemented by Fleming et al. (2005) and further modified by Sathasivan, Fisher and Tam (2008) as follows:

$$BRC = \frac{\mu_m}{k_d} \cdot \left(\frac{free\ \ ammonia\_N}{K_s + free\ \ ammonia\_N} \right)$$ ....………………………..(Eq. 2.10)

μ_m = The maximum specific growth rate of AOB (d^{-1});

Free ammonia= Ammonia (NH_3) and ammonium ($NH4^+$) concentrations (mg-N.L^{-1});

K_s= Half saturation constant for AOB (mg.L^{-1});

k_d = The rate constant for inactivation of AOB by disinfectant (L.d^{-1} mg Cl_2^{-1});

BRC is measured as total chlorine concentration (mg Cl_2 L^{-1}).

Sarker et al. (2013) investigated how temperature (6–35 °C) would impact the biostability parameters in chloraminated water. It was reported that the temperature more than 17 °C is actually controls microorganisms better. Onset of nitrification were noticed above 17 °C due to the reduction of μ_m/k_d value and correspondingly chloramine becomes more effective in controlling nitrification. On the other hand, with increasing temperature decreases the chloramine residual and increases the free ammonia which is suitable for nitrification.

2.4.2 Role of NOM on nitrification

The NOM is a mixture of complex organic compounds that are formed from decomposing plants, animals and microbial materials in soil and water. Total organic carbon (TOC) is often measured as an indicator of NOM. In water distribution systems, they can react with disinfectants to form DBPs. Now DBPs formation has been a global concern on chlorination and chloramination (Rook 1974; Seidel et al. 2005). Many studies (Goslan et al. 2009; Hua & Reckhow 2008; Lu et al. 2009; Shah & Mitch 2011) have reported about the formation of DBPs in chlorinated and chloraminated systems. The impact of NOM on nitrification is not well understood and NOM is not a source for AOB growth (Harrington et al. 2002). However, NOM enhances the growth of nitrifying bacteria by liberating free ammonia as a reaction product.

Consumption and useful effects of the organic carbon on the growth of nitrifiers have been reported by Krümmel and Harms (1982). Organic carbon sources such as pyruvate and peptone increase the cell yield of the *Nitrosomonas* strain, while formate and acetate reduce the lag phase of the *Nitrospira* strain. In chloraminated systems growth of AOB can be indirectly influenced by TOC. It is assumed that TOC could enhance the nitrification by decreasing the chloramine residuals (Zhang, Yan & Edwards 2009).

Although there are some advantages of NOM on nitrification, it could be toxic to the nitrifiers when they live with heterotrophs in chloraminated systems. In the mixed cultured environment, nitrifiers could not win the combat against heterotrophs as they compete for surface area, DO, ammonia and nutrients (Dolinšek et al. 2013; Hockenbury, Grady & Daigger 1977; Sharma &

Ahlert 1977). In the process of producing energy for their cellular functions, nitrifiers must reduce carbon dioxide to cellular carbon which results in lower yield and growth rates compared with the heterotrophs (Rittmann, Bruce E & Manem 1992). Therefore, nitrifiers have a competitive disadvantage with heterotrophs at a high organic carbon level. If there is no organics, heterotrophs do not have substrate to grow, then they must be fully dependant on the cell lysis and the extra cellular substances such as SMP released by nitrifiers for the organic carbon source. This is supported by the finding that at a high organic carbon-to-ammonia ratio, nitrifiers are strongly dominated by heterotrophs (Ohashi et al. 1995; Verhagen & Laanbroek 1991). However, in high DOC levels, chloramine decay promoted by both autotrophs (nitrifiers) and heterotrophs were newly reported. (Herath, Sathasivan & Lam 2015). Therefore, decrease DOC level by reducing NOM from drinking water is extremely important.

2.5 Removal of NOM from drinking water

Ultra filtration

Ultrafiltration (UF) is an established technology in NOM removal in water treatment. In the past two decades it has developed rapidly due to the evolution of both membrane manufacturing design and operation. UF is mainly applied in surface water treatment, filter backwash water treatment and as a pre-treatment for reverse osmosis in desalination (Adham & Gramith 2003). This is mainly applied when the NOM content/ colour is high (>30 mg Pt/l) and turbidity low (<1 NTU) (Ødegaard et al. 2010).

Conventional coagulation/ Floc separation method

This method might be the most commonly used method for the removal of turbidity but some NOM is eventually removed. This method is primarily used to remove the turbidity. Most commonly used coagulants are aluminium sulphate, pre-polymerised aluminium chloride and iron chloride. These processes remove NOM by adsorption onto flocs and can typically achieve 50 - 80% removal of DOC (Parsons et al. 2002)

Oxidation

The colour removal and disinfection is the main target of oxidation. Ozonation normally is the preferred oxidation method. The oxidation of NOM by ozone follow two main pathways; 1) direct oxidation by ozone which selectively targets mainly activated aromatics and double

bonds, and 2) indirect reaction where ozone is decomposed to form hydroxyl radicals which are more powerful but less selective oxidants than ozone (Ødegaard et al. 2010).

Adsorption processes

Granular activated carbon (GAC) is an effective adsorbent used widely for drinking water treatment. Micro pollutants such as pesticides, industrial chemicals, tastes and odours and algal toxins are removed by GAC (Newcombe 1999). The adsorption of compounds to the GAC is influenced by the structural and the chemical characteristics of the carbon surface. (Karanfil et al. 1999; Newcombe 1999). Over the time, it was found that there are bacteria multiply in the GAC filters and contributes to the removal of impurities in the water. After the growth of the bacteria in the granular carbon, it is called biologically activated carbon (BAC). It is also well known that activated carbon filters are used after the ozonation of NOM-containing waters results in increased biological activity in the filter. The run-time of GAC filters increase because of "biological regeneration" (Ødegaard et al. 2010).

Biologically activated carbon (BAC) filters

BAC filtration has been widely applied to remove biodegradable organic compounds (BDOC) similar to sand filtrations (Liu, X et al. 2015). Biological filters eradicate impurities by three main mechanisms: biodegradation, adsorption of micro pollutants, and filtration of suspended solids. Organic matter is consumed by the microbial growth (biofilm) attached to the GAC. Besides, Liu, X et al. (2015) reported that assimilable organic carbon (AOC) also effectively reduced by the pilot scale BAC filters with the time. BAC also can be effective for eliminating synthetic organic chemicals such as benzene, toluene, and pesticides like atrazine which present health concerns. The process also can reduce the concentrations of taste and odour causing compounds such as short-chain aldehydes, amines, aliphatic aldehydes, phenols and chlorinated phenols.

 The DOC removal methods, which have been practised in the drinking water treatment is summarised in the table 2.2.

Table 2. 2: DOC removal methods

DOC removal method	Features	References
Conventional coagulation	Removes organic matter with high MW and low solubility by leaving hydrophilic compounds in the treated water.	Sillanpää and Matilainen (2014), Goslan et al. (2006), García and Moreno (2009), Choi, I-H et al. (2007), Kim, H-C and Yu (2005), Korshin et al. (2009), Dlamini et al. (2013)
Enhanced coagulation	This is improved coagulation conditions, i.e., a low pH level and high dosage of coagulant. This method maximize the removal of DBPs of the precursor	Kabsch-Korbutowicz (2005), Wassink et al. (2011), Zhang, Yue et al. (2015), Fabris, Chow and Drikas (2012).
Adsorption Activated carbon	Widely applies on decolourization, deodorization and organic matter removal including organic micro-pollutants such as algal toxins. pH, temperature, surface texture and ionic strength influence the adsorption efficiency. Maintain required water quality in terms of e.g., DBP formation.	Gonçalves et al. (2013), Li, F et al. (2002), Daifullah, Girgis and Gad (2004), Newcombe and Drikas (1997), Moreno-Castilla (2004), Quinlivan, Li and Knappe (2005).
Oxidation e.g., Ozone	Eliminates taste, odour, colour and certain mineral compounds. Also be used for NOM degradation and microorganisms. O_3 promotes strong oxidation and can oxidize some NOM components directly.	Ghernaout, Ghernaout and Naceur (2011), Molnar et al. (2013).
Membrane • Reverse osmosis (RO) • Nano filtration (NF) • Ultrafiltration (UF) • Microfiltration (MF)	Removal of NOM and contaminants. The NOM properties, including the high MW, negative charge, hydrophobicity and aromatic structure, Higher removal has been reported at a lower pH.	Tijing et al. (2015), Zhao et al. (2011), Gray et al. (2011)
Biological treatment Bio-filtration	Oxidizes the biodegradable fraction of NOM, thus controlling DBPs, trihalomethene (THM) and microbial regrowth. Well-designed bio filtration improves the reduction of precursors of DBPs, bivalent species of iron and manganese.	Korotta-Gamage and Sathasivan (2017), Lautenschlager et al. (2010), Zhang, Yue et al. (2015), Dussert (1994), Dong et al. (2014), Hijnen et al. (2014).
Adsorption (e.g. powdered activated carbon (PAC)) coupled with coagulation	Lower removal rate of low MW organic compounds in the NOM at the coagulation stage. Reduce the coagulant dosage, reduce sludge production.	Zhang, Yue et al. (2015), Wang, Hongtao, Keller and Li (2010), Uyak et al. (2007), Kristiana, Joll and Heitz (2011)
Magnetic ion exchange resin (MIEX®) and coagulation	Removes both organic and inorganic contaminants like sulphate, nitrate or phosphate. Able to remove a fraction of NOM recalcitrant to coagulation. Increase the DOC and DBP precursor reduction.	Jarvis et al. (2008), Metcalfe et al. (2015), Mergen et al. (2008), Morran et al. (2004).
Oxidation (ozonation) and coagulation	Elimination of taste, odour, colour and inactivation of micro-organisms. Produces some hydrophobic neutral and intermediate macro molecules (NOM	Morran et al. (2004), Vilve, M, Hirvonen and Sillanpää (2007), Vilve, Miia, Törönen and

	compounds), which are removed efficiently in the following coagulation process.	Sillanpää (2008), Liu, H et al. (2007), Liu, H, Cheng and Wang (2009).
Advanced oxidation processes (AOPs) and coagulation	Oxidation changes the characteristics of NOM and degrades towards low macro molecule compounds which can be removed easily by coagulation.	Rämö and Sillanpää (2001), Sillanpää, Pirkanniemi and Sorokin (2009), Murray and Parsons (2004), Uyguner et al. (2007).
Membrane Filtration with coagulation	Reduces the amount of coagulant needed while improving turbidity and DOC reductions. Provides an effective hygienic barrier in combination with membranes. Removal of NOM has been significantly affected by the type of coagulant, coagulation conditions, the type of membrane, filtration conditions, and the characteristics of the water to be treated	Blankert, Betlem and Roffel (2007), Kim, Jaeshin, Cai and Benjamin (2008), Cho, Lee and Lee (2006) Choi, YH, Kim and Kweon (2008), Tran et al. (2006), Zularisam et al. (2009).
Adsorption coupled with the membrane (hybrid adsorption)	PAC- increase humic acid, tannic acid and hydrophobic NOM. GAC-not only lower MW and hydrophilic NOM fractions but also for hydrophobic NOM fractions.	Campinas and Rosa (2010),Kim, Jaeshin, Cai and Benjamin (2008), Kim, K-Y et al. (2009)
Ozone coupled with membrane	Direct oxidation can act with activated aromatics and double bonds, leading to higher odour removal and lower TOC removal, while the indirect reaction, in which the O3 is decomposed to hydroxyl radicals that are more powerful but less selective oxidants than O3, can achieve higher TOC removal efficiency.	Song et al. (2010), Karnik et al. (2005)
Adsorption coupled with biological processes ozonation followed by BAC	Aromatic or hydrophobic organic compounds can be converted to more hydrophilic, biodegradable organic compounds, such as aldehydes, carboxylic acids, ketones and other organic acids, which can also be further removed in a subsequent BAC process.	Yapsaklı et al. (2009), (Klymenko, Kozyatnyk & Savchyna 2010), Seredyńska-Sobecka, Tomaszewska and Morawski (2005)

2.6 Protein separation and identification

The key objective of this study is to identify the chloramine decaying microbial products−SMP responsible for chloramine decay. As this microbial product is evidenced as protein/s (Krishna, Sathasivan & Sarker 2012), there were several protein identification techniques implemented in Herath, Torres and Sathasivan (2018). Additionally, a technique has been discovered to concentrate water samples containing chloramine decaying SMP and confirmed that, 30 kDa ultra-centrifugal filters are effective in separating them from nitrified bulk water samples. Ultra-centrifugation is one of the simple method which can be practiced for the size separation and concentration of protein from solutions (Filik & Stone 2009). Therefore, in the chloramine decaying SMP identification process, interested proteins can be narrowed down to 50-30 kDa in size. However, to reduce the complexity of the protein mixture, suitable protein separation method has to be selected and practiced.

2.6.1 Electrophoresis

Electrophoresis is used to separate and compare complex mixtures of proteins (e.g., from cells, subcellular fractions, column fractions, or immunoprecipitates), evaluate purity of a protein during the course of its isolation, and provide estimates of physical characteristics such as subunit composition, isoelectric point, size, and charge (Gallagher 2007). It can also serve to purify proteins for use in further applications. In the electrophoresis, generated electric potential between two electrodes separate charged particles from one another based on differences in their migration speed using a special medium, most often a gel. The most common gels use in the electrophoresis are polyacrylamide and agarose. Polyacrylamide gels typically provide much smaller pores than do agarose gels (Rio et al. 2010). Therefore, Polyacrylamide gels are used typically for the electrophoresis of proteins and relatively small nucleic acids. Polyacrylamide electrophoresis separates macromolecular ions based on a combination of charge, size and shape (Lehrach et al. 1977). Size (and shape) separation is due to the molecular sieving property of the gel.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The most common one-dimensional gel methods utilize the detergent sodium dodecyl sulfate (SDS) to solubilize, denature, and impart a strong negative charge to proteins (Gallagher 2007).

Then the charged, denatured proteins are separated as they migrate based on their molecular mass and ultimately separates proteins. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), also known as one-dimensional gel electrophoresis is the most popular cost-effective method to estimate the molecular mass of protein subunits with considerable accuracy.

Two-dimensional gel electrophoresis (2DE)

Two-dimensional electrophoresis is performed by combining two different electrophoretic methods. Two individually high resolution methods based on isoelectric focusing followed by SDS gel electrophoresis is the most common (Harper, S, Mozdzanowski & Speicher 1998). Isoelectric point and size separation is combined for protein separation and this yields the best separation of complex protein mixtures in comparison to all individual procedures. A larger number of protein bands can be made visible easily with this method and this scale of separation cannot be achieved in the individual methods (Coligan et al. 1995). In the electrophoresis, one-dimensional gels produce rectangular bands while the two-dimensional gels are able to create round or elliptical spots of protein after staining (Harper, S, Mozdzanowski & Speicher 1998).

2.6.2 Mass spectrometry (MS)

Since the development of ionisation methods compatible with large, nonvolatile biomolecules, mass spectrometry (MS) has become the most useful technique for protein characterisation, identification, and quantitation. Mass spectrometry is a technique in which gas phase molecules are ionized and their mass-to-charge ratio is measured by observing acceleration differences of ions when an electric field is applied. Lighter ions will accelerate faster and be detected first. If the mass is measured with precision, then the composition of the molecule can be identified. In the case of proteins, the sequence can be identified. Most samples inserted into the mass Spectrometry are a mixture of compounds. A spectrum is acquired to give the mass-to-charge ratio of all compounds in the sample. Mass spectrometry is also known as 'mass spec' or MS for short. Mass spectrometry throws light on molecular mechanisms within cellular systems. It is used for identifying proteins, functional interactions, and it further allows for determination of subunits. Other molecules in cells such as lipid components can also be defined.

A mass spectrometer is composed of several different parts: a source that ionizes the sample, the analyzer that separates the ions based on mass-to-charge ratio, a detector that "sees" the ions, and a data system to process and analyze the results. Relative abundance of an ion can also be measured using mass spectrometry. Different compounds have differential ionization capabilities and therefore intensity of the ion is not a direct correlation to concentration

2.7 The microbial community in chloraminated water distribution systems

The majority of chloramine loss in chloraminated systems occurs due to the chloramine decaying SMP and the microbes occupied in the nitrified conditions. There were several investigations have been carried out to characterize and quantify nitrifiers, especially AOB in chloraminated systems. AOB are slow growing autotrophic (use carbon dioxide to support their growth), aerobic bacteria that occupy in a vast range of aquatic environments. Due to the high energy consumption for fixing inorganic carbon, nitrifiers show growth rates are slow (Rittmann, Bruce E 1984). Particularly, in chloraminated drinking water distribution system, to some extent AOB may have chloramine resistance. Further, laboratory inactivation experiments also proved the necessity of long time or high chloramine residuals for inactivating nitrifiers (Cunliffe 1991; Oldenburg et al. 2002).

There could be difference in AOB species found in different environments. Furthermore, Claros et al. (2010) reported that AOB species are different in lab and pilot-scale systems. Dominant AOB in biofilms and bulk waters of pilot and full-scale chloraminated system are *Nitrosomonas* spp., members of the *Betaproteobacteria*, characterised using the 16S rRNA gene-targeted terminal restriction fragment length polymorphism (T-RFLP) and clone library. *Nitrosospira* spp., another subgroup of *Betaproteobacteria* was found as a small fraction of the AOB in the same systems (Lipponen et al. 2004; Regan, John M et al. 2003; Regan, John M, Harrington & Noguera 2002). It is also confirmed both *Nitrosomonas* spp. and Nitrosospira spp. are the common strains, they regularly present in the chloraminated distribution systems (Hoefel, Daniel et al. 2005). *Nitrospira* spp. were identified in several studies as the largest subgroup of NOB using the 16S rRNA gene clone library (Martiny et al. 2003) and 16S rRNA gene-targeted T-RFLP (Regan, John M et al. 2003; Regan, John M, Harrington & Noguera 2002). *Nitrobacter* spp. NOB was also detected in chloraminated systems (Noguera et al. 2009; Regan, John M et al. 2003; Regan, John M, Harrington & Noguera 2002).

Besides the nitrifying bacteria, nitrification can be carried out by some heterotrophs such as *Alcaligenes faecalis*, *Pseudomonas*, *Thiosphaera pantotropha*, fungi, and some algae (Bock et al. 1992; Watson et al. 1989). Nevertheless, the rate of nitrification is slower than the autotrophic nitrifiers. Apart from the bacteria, Ammonia-oxidizing *Archaea* (AOA) identified in a marine environment and in wastewater treatment plants can carry out nitrification (Könneke et al. 2005; Park et al. 2006). Recently, Woolpunda distribution system of South Australia have reported the presence of AOA (Hoefel, D 2011) in their chloraminated system.

Almost all of these studies were carried out on the community of nitrification bacteria. There were limited studies had been accompanied to distinguish the overall microbial community in chloraminated systems. For the first time, heterotrophic bacterial community in a chloraminated system were reported by Williams et al. (2004). Dominance of *Alphaproteobacteria* was recognized in both model chloraminated and chlorinated water distribution systems. Sequences aligned with *Alphaproteobacteria* including *Afipia*, *Sphingomonas*, *Brevundimona*s, *Blastomonas*, *Hyphomicrobium*, *Methylocystis* and *Bradyrhizobium* were reported in the systems. Furthermore, *Betaproteobacteria* was more abundant in the chloraminated water than in the chlorinated water (Williams et al. 2004).

Despite the detailed investigation of bacterial communities in many chloraminated systems, investigation of the microbial community with respect to chloramine residual or nitrification stages was largely missing. Krishna, Sathasivan and Ginige (2013) for the first time revealed the impact of decaying chloramine residuals and associated nitrification on microbial communities in chloraminated distribution system. It was done by setting up a lab scale reactor system simulating a full scale chloraminated water distribution system. The study reported that some microorganisms appear to resist chloramine concentrations commonly maintained by utilities in the distribution systems. Microbial communities changed with changing chloramine residuals and nitrification metabolites and diversity of them increased with decreasing chloramine residuals. Bacterial classes *Solibacteres, Nitrospira, Sphingobacteria, Betaproteobacteria* were the dominant when the reactor content experiences low chloramine residuals (< 0.65 mg.L^{-1}) and high nitrification activities. Classes *Actinobacteria* and *Gammaproteobacteria* were dominant in high chloramine residual ($1.60 - 2.18$ mg.L^{-1}) maintained reactors. The class *Alphaproteobacteria* was present in all chloramine residuals ($0.03 - 2.18$ mg.L^{-1}) irrespective of variation in nitrification activities. Nitrifiers and biologically induced ammonia oxidation only took place once chloramine had decreased below 0.65 mg.L^{-1} and well known AOB with two novel species were identified.

In both chloraminated and chlorinated systems, several EPS and SMP producing bacterial strains have been identified previously. It is essential further investigating EPS/SMP producing microbes to control accelerated chloramine decay due to these microbial products. When biofilms are employed as part of a water or wastewater treatment strategy, SMP will be released (Carlson & Amy 2000). Interestingly, nitrifiers produce EPS and/or SMP (Kindaichi, Ito & Okabe 2004; Ohashi et al. 1995; Rittmann, Bruce E, Regan & Stahl 1994; Sepehri & Sarrafzadeh 2018; Shi, Y et al. 2018) which mainly consist of polysaccharides, proteins, nucleic acids and lipids (Flemming & Wingender 2010; Sheng, Yu & Li 2010) in certain scenarios. Other than nitrifiers, heterotrophic bacteria such as, *Mycobacteria* (Chapman 2003) and *Methylobacterium* (Tsagkari & Sloan 2018) produce EPS in drinking water system.

On the other hand, NOM content of the water greatly impact on nitrification in the water distribution systems. It could host different types of microbial community with the variation of NOM content in the distribution systems. Besides, SMP accelerated the chloramine decay is identified in nitrified chloraminated systems. Studying the microbial community changes in different NOM could lead to recognize the responsible bacteria for the production of SMP. It could hint for employing measures to control SMP associated chloramine decay in chloraminated distribution systems. This would facilitate better opportunities for controlling the SMP producing bacterial population rather than controlling the nitrification.

2.8 Strategies in prevention and control of chloramine decay

Nitrification has to be controlled for cutting down the associated accelerated chloramine decay in the distribution systems. This will lead to maintain the water quality in the distribution system by controlling the bacterial growth. There are several strategies to control the nitrification in chloraminated systems.

- **Increasing chlorine to ammonia ratio**

Seidel et al. (2005) reported that optimizing the chlorine to ammonia ratio is the most common nitrification control technique. In this technique nitrification is controlled by reducing the free ammonia level (Kirmeyer 2004; Lee, CO et al. 2014). Nevertheless, nitrifiers can grow even with small amount of ammonia and therefore is a less effective method. Moreover, there is a chance of dichloramine formation while maintaining chloramine at higher chlorine to ammonia ratio (5:1), results taste/odour problems and higher DBPs formation (AWW 2006).

- **Increase chloramine residual**

In the long term approach increasing chloramine residual by adding free chlorine may be an effective nitrification control measure, but once nitrification triggers it is almost impossible to stop it even with high chloramine concentration of 8 mg.L^{-1} (Skadsen 1993). In South Australia, nitrifiers were found in samples having more than 5 mg.L^{-1} of monochloramine (Cunliffe 1991). It was also reported that the nitrification didn't decrease in most pipe materials until the chloramine level was increased to 4 mg.L^{-1} and maintained at the same level for several weeks (Zhang, Yan & Edwards 2009). Once the onset of nitrification started, it is almost impossible to stop as increased nitrite concentration decay chloramine even after introduction of high chloramine dose.

- **Breakpoint chlorination**

Breakpoint chlorination is one of the most effective control method for inactivating nitrifiers (Kirmeyer 2004). It is applied in several water utilities for a period of one week to a month once or twice a week. Increase DBPs concentration, unaccepted chlorinous taste ((Ferguson, DiGiano & Chandran 2005; Harms & Owen 2004), extensive labour involvement and usage of massive amount of chlorine at initial stage are some negative effects of break point chlorination. Krishna, Sathasivan and Kastl (2014) reported that, compounds (e.g., SMP) responsible for accelerated chloramine decay cannot be destroyed by repeated rechloramination (just enough to satisfy breakpoint chlorination demand from ammonia and nitrite oxidation). Moreover, chlorine might completely decay before it gets to deactivate nitrifiers.

- **Flushing (decrease water age)**

This is a short duration effective method of controlling nitrification. In this method nitrifiers could be flushed from the system. However, with the time they could re-establish between flushing intervals. Flushing can also remove tubercles and sediments, thus disinfectant can penetrate into biofilms which contain nitrifiers (Harms & Owen 2004).

- **Removal of organic matter**

As NOM accelerate the decay of chloramine, it is an effective method of controlling nitrification by removing NOM at the treatment plant which is commonly practised in Europe. In a pilot scale study, Harrington et al. (2002) reported that nitrification could be avoided or prolonged the onset time by maintaining total chlorine residual above 2.2 mg.L^{-1} and the

chlorine to free-ammonia ratio greater than 1.9. They also observed that the onset of nitrification was stopped in high TOC removed samples during a four-day retention time. Further, no nitrification events were observed when the retention time was reduced to one day. Removal of NOM also controls the heterotrophic bacterial growth in the distribution system.

- **pH adjustments**

Nitrification occurred over a wide pH range (from 6.5 to 9.5). However, many studies reported the optimum pH condition for nitrifying bacterial growth as pH 7.5-8.5 (Grunditz & Dalhammar 2001; Odell et al. 1996; Painter 2013; Wilczak et al. 1996; Wolfe & Lieu 2003). Increase the pH level more than 8 could help in reducing nitrifiers growth and chloramine decay. Eventually, it reduces the chloramine inactivation effect.

- *F_m* **Method**

F_m method was revealed to maintain a sufficient chloramine residual by minimizing microbial acceleration of chloramine decay in chloraminated water reservoir of Sydney Water Distribution System, Australia (Sathasivan, Fisher & Kastl 2010). This provides the precise values of chloramine stability by calculating the decay rate coefficients (chemical and microbial) with monitoring chloramine residual. Therefore, the early warning of onset of nitrification (Fisher et al. 2009; Sathasivan, Fisher & Kastl 2010) can be identified and control strategies for maintaining the chloramine residual can be applied.

- **Addition of inhibitors**

sodium chlorite

Several studies reported the application of chlorite as nitrification inhibitor and therefore maintain the recommended chloramine level in the distribution systems (Gagnon et al. 2005; Karim & LeChevallier 2006; McGuire, Lieu & Pearthree 1999; Rahman, Encarnacion & Camper 2011). McGuire, Lieu and Pearthree (1999) hypothesized that chlorite can stop or exclude the potential nitrification. It is also reported that short term application of chlorite ion (0.2 mg.L^{-1}) is effective in preventing nitrification in full-scale distribution system (McGuire et al. 2006). Nevertheless, chlorite could be converted to chlorine dioxide in an acidic environment that could create by oxidation of ammonia during nitrification by biofilm on the surfaces (Svecevičius et al. 2005). Chlorite could be toxic if applies in high levels (Harms & Owen 2004).

Heavy metals

Addition of copper and silver as inhibitors for controlling nitrification has been attempted in the research (Sathasivan, Fisher & Kastl 2005; Zhang, Yan, Love & Edwards 2009). Sarker (2012) reported that copper concentrations greater than or equal to 0.25 mg-Cu.L^{-1} intensely withdrawn the AOB activities (nitrite production) in severely nitrifying chloraminated waters. Considering those results it was proved that copper alone or with chloramine can inhibit nitrification hence can help in sustaining chloramine residual in the distribution system. The inhibitory effect on pure cultured nitrifiers of selected metals are shown in Table 2.3. However, effect on mixed culture environment could be significantly difference.

Table 2. 3: Inhibition range of various metals

Metal	Concentration range (specific inhibiting extent)	Culture purity (growth condition)	Reference
Cu (II)	0.01 ppm had 5% inhibition	Wastewater treatment inoculum	Waara and Wilander (1985)
	>0.4 ppm	Not specified	Martin and Richard (1982)
Ni (II)	>0.1 ppm	Not specified	Martin and Richard (1982)
	0.7 ppm	Activated sludge	Harper, SC et al. (1996)
	1 ppm had 20% inhibition	Activated sludge	Ibrahim (2018)
Cr	0.3 ppm Cr (III)	Activated sludge	Harper, SC et al. (1996)
	>1 ppm (II)	Not specified	Martin and Richard (1982)
	1 ppm had 10 % inhibition	Activated sludge	Ibrahim (2018)
	>0.3 ppm Cr (III)	High ammonia, Low Biodegradable organic Leachate	Harper, SC et al. (1996)
Zn (II)	3 ppm had 80% inhibition	Wastewater treatment	Waara and Wilander (1985)
	11 ppm had 25% inhibition	Activated sludge	Martin and Richard (1982)
	>10 ppm	Not specified	Martin and Richard (1982)
Pb (II)	>0.5–1 ppm	Not specified	Martin and Richard (1982)
	>1 ppm, 1.7 ppm had 90% inhibition	Wastewater treatment Inoculum	Waara and Wilander (1985)
Cd (II)	<75 ppb had <10% inhibition, 500 ppb had 85% inhibition	Wastewater treatment inoculum	Waara and Wilander (1985)
	14.3 ppm had 42% inhibition	Activated sludge	Martin and Richard (1982)

Krishna, Sathasivan and Sarker (2012) reported that the accelerated chemical and microbial chloramine losses were significantly reduced after dosing silver at a concentration of 0.1 mg-Ag.L^{-1} to the nitrified water samples obtained from laboratory scale system. Silver has been used for water disinfection and is effective inactivation or killing of planktonic bacteria (Kim,

Jaeeun et al. 2004; Silvestry-Rodriguez et al. 2008). Zhou et al. (1997) reported that silver does not react with most of NOM in distribution system by resulting minimal possibility of producing toxic by-products. With advantages of employing silver in the distribution system; more investigation has to be carried out for optimizing the silver dose, dosing location and time in the distribution system.

According to the literature survey, it is indispensable to control chloramine decay in drinking water distribution systems for supplying better quality drinking water to the consumers. However, numerous essential factors on chloramine decay such as CDP, impact of DOC on CDP and heterotrophic bacteria have not been further investigated and reported. Therefore, in this study several experiments and protein identification techniques were employed to identify the protein(s) and micro-organism(s) believed to be accountable for accelerated chloramine decay. Furthermore, this study investigates the effect of NOM variations on different nitrifying conditions and the related bacterial community followed by the impact of NOM on CDP. Silver is one of the effective disinfectant controlling micro-organisms in water environment. However, there are limited information of silver application on nitrifying bacteria and CDP control along with the accelerated chloramine decay. Hence, effect of silver on nitrifying micro-organisms and CDP were acknowledged in this study.

Chapter 3

Materials and Methods

This chapter includes a detailed description of the material and methods such as laboratory scale reactor maintenance and operation, feed water preparation employed throughout this dissertation. Each subsequent chapter has a smaller, abridged materials and methods section, but the current chapter should be the reference for duplicated experiments. Furthermore, common analytical procedures and stock chemical solution preparation practised for the conducted experiments all through the dissertation were discussed.

3.1 Analytical procedures

Total chlorine (TCl), total ammoniacal nitrogen (TAN), nitrite, and nitrate were measured immediately after sample collection. TAN is the summation of NH_3-N, NH_4^+-N, and nitrogen associated with chloramines. A Gallery (Thermo Scientific) high-precision wet chemistry automated analyser was adopted for measuring TAN, nitrite, and NO_x (nitrite + nitrate) concentrations, which performs discrete, spectrophotometric analysis with an optical multi-cell cuvette.

TAN concentration was measured following the salicylate ion method. Available ammonia reacts with hypochlorite ions that are generated by the alkaline hydrolysis of sodium dichloroisocyanurate to form monochloramine, which reacts with salicylate ions in the presence of sodium nitroprusside at approximately pH 12.6 to form a blue compound. The compound can be spectrophotometrically identified at 660 nm. Nitrite is measured by reacting

it with sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride to form a coloured azo-dye. Thus, absorbance is measured spectrophotometrically at 540 or 520 nm (APHA 1998). In a mixture of nitrite and nitrate, nitrate is measured by catalytically reducing nitrate into nitrite ions using a nitrate-reductive enzyme in the presence of reduced nicotinamide dinucleotide). The total nitrite ions are then measured following the sulphanilamide method and reported as NOx. Nitrate levels are determined by subtracting nitrite from NO_x. The analyser has a TAN and nitrite-N detection limit of 0.002 mg.L^{-1} and NO_x detection limit of 0.005 mg-N.L^{-1}. Standard curves for TAN, nitrite, and NO_x were calibrated between 0.0 and 1.0 mg-N.L^{-1} using stock ammonium chloride, sodium nitrite, and sodium nitrate solutions, respectively. The experimental error for TAN and nitrite measurement was 2%, but the error for NO_x measurement was 5%.

TCl residuals were measured following the DPD colourimetric method using a HACH pocket colourimeter (HACH DR 1900). It was assumed that over 99% of the chloramine was present as monochloramine above pH 8.0, and the Cl to TAN mass ratio was approximately 4.0 or less (Valentine, R et al. 1987). The TCl measurement had an experimental error of ±0.03 mg-Cl$_2$.L^{-1}. A portable pH meter (HACH 40d Multi) was used to measure the pH with a measurement error of ±0.1.

Total organic carbon (TOC) and dissolved organic carbon (DOC) were measured using a Shimadzu Total Organic Carbon Analyser (TOC-L CSH/CSN), Japan. (with an experimental error of ±5%). Before measuring DOC, the samples were filtered through pre-washed 0.45 µm polycarbonate membrane filter papers. After placing the filter paper on the filtration apparatus, approximately 100 mL of Milli-Q water was passed through the device to minimise contamination into the sample by DOC from the filter paper.

3.2 Preparation of stock chemical solutions

Stock solutions of all chemicals were prepared using analytical-grade chemicals diluted/ dissolved in Milli-Q water. The expected monochloramine concentration in feed water and in decay tests was achieved using 1 g-N.L^{-1}ammonium chloride and 1 g-Cl$_2$.L^{-1} sodium hypochlorite solutions which were prepared from 99.998% NH$_4$Cl powder and 12.5 % sodium hypochlorite solution, respectively. The alkalinity levels of feed water were maintained using 8 g-HCO$_3^-$.L^{-1} sodium bicarbonate solution prepared from ≥ 99.9 % NaHCO$_3$ powder.

Anticipated silver concentration in decay tests was achievd using 1 g-Ag.L.$^{-1}$ silver nitrate solution prepared from $\geq$ 99 % AgNO$_3$ salt. To adjust the pH, 1 M sulfuric (H$_2$SO$_4$) acid and sodium hydroxide (NaOH) solutions were prepared from 99.999 % H$_2$SO$_4$ viscous liquid and $\geq$ 98 % NaOH pellets, respectively. Nitrite concentration in samples utilised for conducting of chloramine decay tests was adjusted to the same level using a stock solution of 1 g-N.L^{-1} sodium nitrite (NaNO$_2$) prepared from $\geq$ 99.0 % NaNO$_2$ crystalline powder.

3.3 Maintenance of lab scale reactors connected in series

3.3.1 Feed water sample collection and feed water preparation

Sydney Water Corporation supplies water to approximately 4 million customers. The Orchard Hills Water Filtration Plant (WFP) treats about 70 million litres of drinking water per day for supplying drinking water to suburbs in Penrith area. Water mainly from Warragamba Dam is treated by coagulation/ flocculation/ filtration, followed by disinfection. From the Orchard Hills water filtration plant, 1.0 m^3 of filtered water was collected before the disinfectant was added, transported to Western Sydney University, and stored in a high-density polyethylene (HDPE) tank at room temperature (18–23 °C). To eliminate contamination by indigenous microbes in the feed water, chlorine (from the stock 1 g-Cl$_2$.L^{-1} sodium hypochlorite solution) was added to achieve higher chlorine level (around 0.3 mg-Cl$_2$.L^{-1} higher) than the required level (about 2.2 to 2.3 mg Cl$_2$.L^{-1}) at least 3 h before ammonia addition (from a stock 1 g-N.L^{-1} ammonium chloride solution). If the chlorine level decreases below the required level at the time of ammonia dosing, an additional amount of chlorine was added to achieve the required chlorine level. This was kept in a 20 L HDPE feed water tank. The feed water was prepared every four days to feed the series of reactors. The TCl to TAN mass ratio of 4:1 was maintained to minimise the free ammonia residuals. The feed water TOC level varied according to the experimental setup. For The pH in the feeding tank was subsequently adjusted to 8.0± 0.1 to provide the optimum condition for monochloramine formation.

To prepare feed water containing 0–1 mg.L^{-1} DOC level, the collected water was fed through granular activated carbon (GAC) as described in Section 3.3.4. Water containing 2-3 mg.L^{-1} DOC concentration was prepared by diluting 4-5 mg.L^{-1} DOC containing feed water (1:1) with Milli-Q water. before the reactors were fed pH in the feed tank was subsequently adjusted to 8.0±0.1.

3.3.2 Laboratory scale system set-up

The laboratory-scale systems were set up at the Environmental Engineering Laboratory in Western Sydney University. The systems (Fig. 3.1) contained five reactors (R-1, R-2, R-3, R-4, and R-5) that were connected in series through HDPE pipes. Each 5 L reactor with a lid and the feed water tank were composed of HDPE. The automatic water flow control devices controlled the water flow rate in the system between feeding tank and R-1. Further, the outlet and inlet pipe levels of other reactors were adjusted to create gravitational flow along the system. Water samples were regularly collected from the outlet for analysis, which was fixed at the bottom of each reactor.
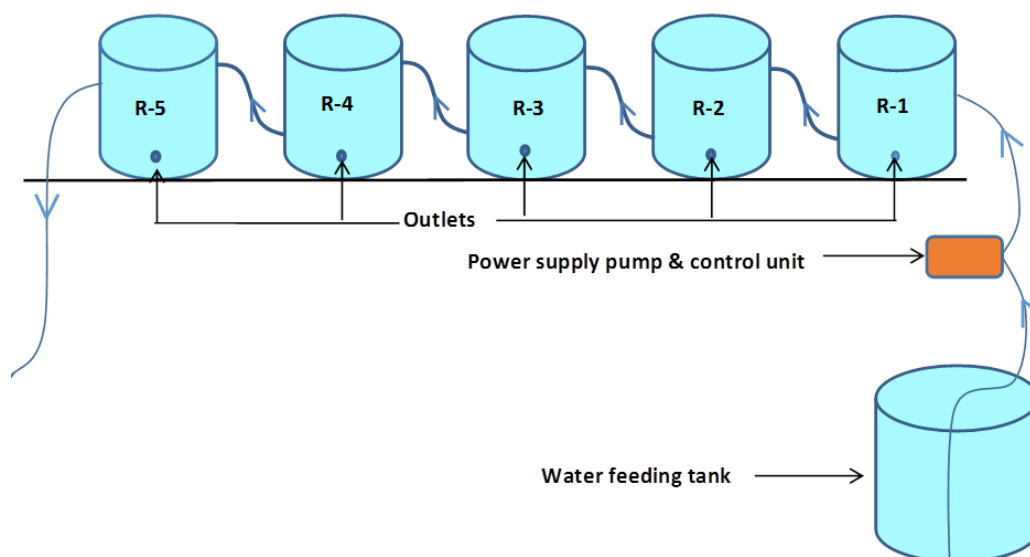
Figure 3.1: Schematic diagram of laboratory scale system setup. Each reactor was 5 L in volume and the feed water tank was 20 L. Arrow heads indicate the direction of gravitational water flow with a retention time of 24 h. All tanks and pipes were made of HDPE and closed by a lid (HDPE).
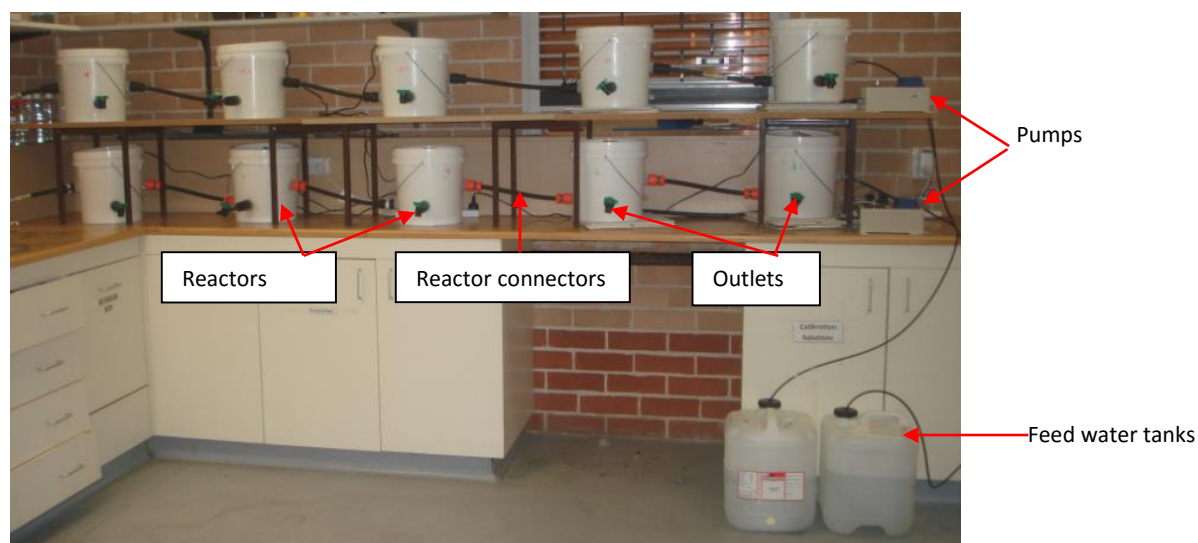
Figure 3.2: Photograph of two laboratory scale systems

3.3.3 Operation of the laboratory scale system

The reactor sets were operated in a room with a temperature of 20–23 °C. During the start-up period, feed water was prepared with a chloramine concentration of approximately 1.10 mg-$Cl_2.L^{-1}$ (TCl:TAN mass ratio, 4:1). To achieve 1.10 mg.L^{-1} chloramine concentration in the feed water, initial chlorine concentration was setup at 1.4 mg-$Cl_2.L^{-1}$ (i.e. 0.3 mg.L^{-1} more than the required 1.10 mg.L^{-1}). After the chlorine concentration dropped down to 1.10 mg-$Cl_2.L^{-1}$ in 3 h time period, feed water ammonia concentration was brought up to 0.28 mg-N.L^{-1} to achieve TCl:TAN mass ratio at 4:1. Approximately 5 L of water was fed continuously per day, and the water volume was maintained in each reactor to achieve a retention time of 24 h. Different nitrification conditions (from no to severe nitrification), which generally occur in a full-scale distribution system (Sathasivan, Fisher & Tam 2008) were created along the reactors by maintaining 24 h retention time. To facilitate nitrification and acquire a specific inoculum that is usually present in a distribution system, chloraminated water samples were collected from the Sydney Water distribution system under different nitrifying conditions. A mixture of the water samples was placed as a seed for micro-organisms in each reactor, excluding R-1. The lab-scale reactor system was continuously operated during the experimental period. Gradually, chloramine concentrations in the feed water tanks were increased to 2.10-2.30 mg-$Cl_2.L^{-1}$,

which were maintained until the experiment was completed. Parameters such as TCl residuals, TAN, nitrite, and nitrate were continuously monitored along the reactors (R-1 to R-5).

3.3.4 GAC column maintenance

To reduce the DOC content of the feed water to 0–1 mg-C.L^{-1}; an activated carbon column was separately maintained. The granular size of the activated carbon was 2.36 to 4.65 mm, and the volume of the column was 160 mL. Water was continuously fed by the pump to maintain a retention time of 40 min. The temperature was maintained at 20–23 °C. It is possible that some microbiological activities could occur in the column due to the indigenous microbes of the water collected from the treatment plant.

The GAC column was effective at removing organic carbon, but heavy metal removal could not be confirmed, although it is assumed that some removal is possible (Korotta-Gamage & Sathasivan 2017). Heavy metals, such as copper, can inhibit or stimulate the activity of nitrifying bacteria (Sarker, Sathasivan & Rittmann 2015). As the column was operated over a long period, it inevitably behaved as biologically-activated carbon.

3.4 Maintenance of free standing reactors

3.4.1 Reactor operation

Two sub-reactors were operated at 20 – 23 °C. The reactors were made of glass and each had a capacity of 5 L. Reactors were fed daily with nutrient solution prepared in Milli-Q water (DOC<0.1 mg-C·L^{-1}) containing all the necessary inorganic elements. During the first five days of the operational period, 1 L of reactor water was replaced manually with 1 L of feed water (nutrient solution), daily. The chloraminated reactor was fed with nutrient solution to which chloramine (0.85 mg·L^{-1} chlorine) and ammonia (0.6 mg-N·L^{-1}) were added at a Cl-to-TAN mass ratio of 1.4:1. The ammoniated reactor was fed with the nutrient solution to which was added only ammonia (0.6 mg-N·L^{-1}). The seed matter (DOC: 4.5 mg-C·L^{-1}, chlorine: 0.35 mg-Cl$_2$·L^{-1}, TAN: 0.170 mg·L^{-1} and nitrite: 0.207 mg-N·L^{-1}) was obtained from the severely nitrified reactor maintained in a separate continuously fed laboratory-scale reactor set (described in section 3.3.2).

After the first five days, 2 L of water within each reactor was replaced with feed water on alternate days. At the time of adding the nutrient solution, the chloramine concentration inside the chloraminated reactor was reset to 0.45 mg-$Cl_2 \cdot L^{-1}$ (Cl-to-TAN mass ratio of 1.3 :1). That is, the chlorine and ammonia concentrations were measured and amounts of ammonia and chlorine sufficient to reach 0.45 mg.$Cl_2 \cdot L^{-1}$ were added. Similarly, in the ammoniated reactor, the ammonia concentration inside the reactor was reset to 0.35 mg-$N \cdot L^{-1}$. The reactor contents were thoroughly mixed by shaking before draining and after feeding. Parameters such as TCl residuals, TAN, nitrite and nitrate were continuously monitored in both reactors.

3.4.2 Feed water (nutrient solution) preparation

The feed water was prepared using Milli-Q Water to provide the necessary nutrients for microbial growth. The feed water contained $MgSO_4 \cdot 7H_2O$ (40 mg$\cdot L^{-1}$); KH_2PO_4 (100 mg$\cdot L^{-1}$); $CaCl_2$ (15 mg$\cdot L^{-1}$) and a trace elements solution (1.0 mL$\cdot L^{-1}$). The trace elements solution was prepared as described by Lipponen et al. (2004), but Na_2EDTA (Titriplex III) was excluded to maintain a low DOC concentration in the feed water. To provide an inorganic carbon source and to maintain alkalinity, 30 mg$\cdot L^{-1}$ $NaHCO_3$ was added to the feed water. The feed water pH was adjusted to 8.0±0.1. The resultant solution is herein termed 'nutrient solution'. Depending on the reactor (ammoniated reactor or chloraminated reactor), total chlorine or ammonia was added to the nutrient solution to prepare the feed for each reactor.
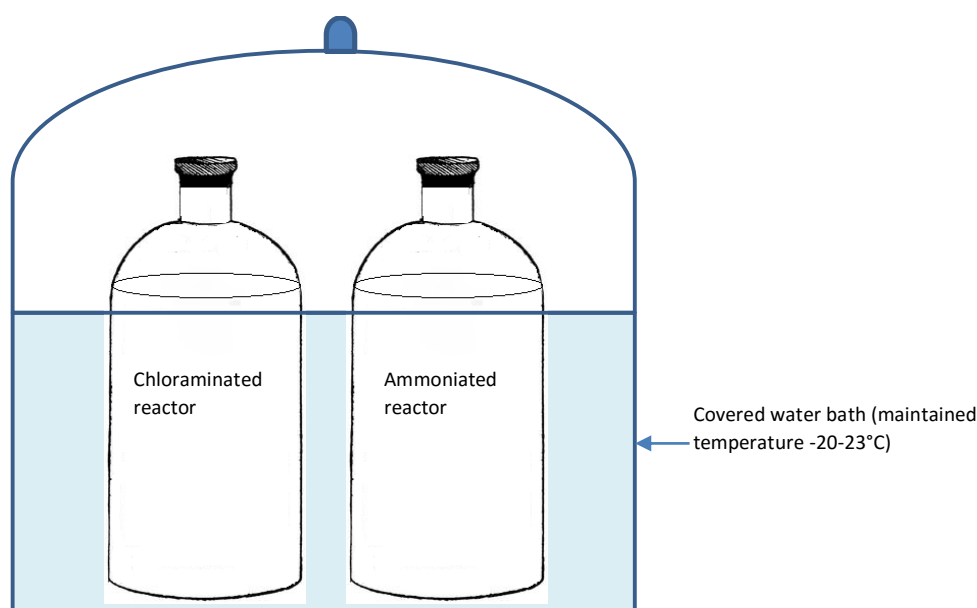
Figure 3.3: Chloraminated and Ammoniated reactor maintenance

Chapter 4

Effects of feed water NOM variation on chloramine demand from chloramine-decaying soluble microbial products during rechloramination

4.1 Introduction

Disinfectants are used to limit the prevalence of waterborne diseases and deactivate pathogenic organisms in water supplies. From the early 1900s, a small town in Belgium started to use disinfectant in water treatment (White, G 1999). Chlorine gas and reagents have been the most common forms of drinking water disinfectant for nearly a century. Due to fast decay and the high formation of regulated disinfection by-products, alternative secondary disinfectants have received increasing attention. Chloramine is the second most popular disinfectant used in many countries, such as the USA, Great Britain, South Africa, France, Canada, and Australia.

Chloramines are chemical compounds that contain chlorine and ammonia. The specific species of chloramine used in drinking water disinfection is monochloramine, as it is more chemically stable than chlorine. Despite its benefits, maintaining sufficient chloramine levels in chloraminated systems throughout the year is a challenge due to chemical and microbial decay in warmer waters.

Chemical reactions that decay chloramine include auto-decomposition (Eq. 4.1) (Vikesland, Valentine & Ozekin 1996), nitrite oxidation (Eq. 4.2) (Vikesland, Valentine & Ozekin 1996), and the reaction with natural organic matter (NOM) (Eq. 4.4) (Vikesland, Ozekin & Valentine 1998). Reaction Eq. 4. 3 (Vikesland, Valentine & Ozekin 1996) is also reported to occur in parallel with the auto-decomposition reaction (Eq. 4.1), but it is supposedly insignificant. The reaction with NOM (Eq. 4.4) is primarily via redox and substitution reactions (forms ammonia chloride and oxidized organics (NOM_{ox}), but the formation of N_2 gas through auto-decomposition reaction (Eq. 4.1) also simultaneously occur in NOM containing waters (Duirk et al. 2005; Vikesland, Ozekin & Valentine 1998). Both NH_2Cl and $HOCl$ (formed via disproportionation of NH_2Cl) react in parallel to oxidise NOM (Duirk et al. 2005; Vikesland, Ozekin & Valentine 1998).

$$3NH_2Cl \longrightarrow N_2 + NH_3 + 3Cl^- + 3H^+ \qquad \text{(Eq. 4.1)}$$

$$NH_2Cl + NO_2^- + H_2O \longrightarrow NO_3^- + NH_4^+ + Cl^- \qquad \text{(Eq. 4.2)}$$

$$4\,NH_2Cl + 3H_2O \longrightarrow 3NH_3 + NO_3^- + 4Cl^- + 5H^+ \qquad \text{(Eq. 4.3)}$$

$$NOM + NH_2Cl \longrightarrow NOM_{ox} + NH_4^+ + Cl^- \qquad \text{(Eq. 4.4)}$$
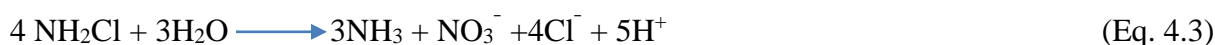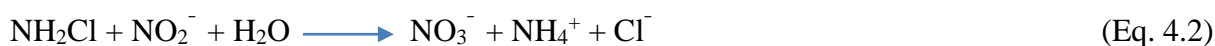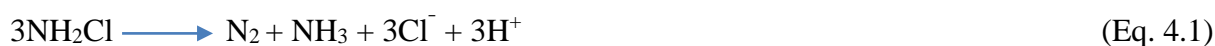
Nitrifying organisms, especially ammonia-oxidising microbes (AOM), can decay chloramine by the production of nitrite, which eventually is oxidised to nitrate by chloramine (Eq. 4.2). Changes in NOx-N (nitrite-N + nitrate-N) indicate the activity of AOM, as nitrite-oxidising microorganisms, another group of nitrifying microbes, could also oxidise nitrite to nitrate.

Questioning the traditional belief that nitrite from nitrification is the sole microbial contributor to microbial chloramine decay, microbial decay factor method was developed to separate the microbial and chemical reactions (Sathasivan, Fisher & Kastl 2005). Upon application of the method in many samples from full-scale systems, microbial chloramine decay has been observed even before the onset of nitrification (Herath, Sathasivan & Lam 2015; Sathasivan, Fisher & Tam 2008) After nitrification begins, decay is approximately five times higher than that experienced before it began (Sathasivan, Fisher & Tam 2008). They termed this as severe nitrification. Under these conditions (nitrite > 0.02 mg-N.L^{-1}; decay coefficient > 0.006 hr^{-1}), contrary to the traditional belief that nitrite was solely responsible for acceleration, higher decay than explained by nitrite was experienced (Krishna & Sathasivan 2010). Krishna,

Sathasivan and Sarker (2012) provided indirect evidence that the activity of soluble microbial products (SMP) could be the major factor in accelerating chloramine decay in filtered severely nitrifying water (some of these results are reproduced in Fig. S1). The potential presence of SMP is consistent with previous findings in wastewater where SMP could be measured under nitrifying conditions (Furumai & Rittmann 1992; Rittmann, Bruce E, Regan & Stahl 1994). However, it should be noted that SMP can also be produced under non-nitrifying conditions (Korotta-Gamage & Sathasivan 2017) such as in biological activated carbon systems and hence cannot completely allocate SMP accelerating chloramine decay to only that from nitrifiers, as heterotrophs are found in chloraminated systems under mild and severely nitrifying conditions (Krishna, Sathasivan & Ginige 2013; Regan, John M, Harrington & Noguera 2002).

Sathasivan and Bal Krishna (2012) could explain the reduction in residual chloramine through auto-decomposition (Eq. 4.1) and nitrite oxidation by chloramine (Eq. 4.2). Krishna, Sathasivan and Sarker (2012) demonstrated that the potential SMP catalyses (i.e., upon repeated re-chlorination of filtered severely nitrifying samples, the same decay rate of chloramine associated with a rapid loss of total ammoniacal nitrogen (TAN) and nitrite persisted for the same initial conditions) both nitrite oxidation (Eq. 4.2) and auto-decomposition (Eq. 4.1, Fig. A.1 and Fig. A.2). Various protein degeneration treatments (silver, high temp, high/low pH) could inhibit the chloramine decay acceleration activity of SMP (Aune & Tanford 1969; Dolgikh et al. 1981; Kingston & Haswell 1997; Kronman, Holmes & Robbins 1971; Lew et al. 2002; Li, Q et al. 2008). While these results indicated the presence of protein(s) within the severely nitrified water sample, it is yet unclear if the causative agents were protein(s).

A wide molecular weight (MW) range of SMP has been reported. The majority of SMP have a MW greater than 10 kDa (Jarusutthirak & Amy 2007), as do proteins (Haurowitz & Koshland 2017). However, most of the organic compounds within NOM are below 10 kDa in size (Amy et al. 1992). Ultra-centrifugation with a higher MW cut-off (>10 kDa) could be used to separate SMP from solutions containing NOM (Filik & Stone 2009).

The impact of potential SMP (hereafter referred to as chloramine-decaying SMP (cSMP)) could not be always observed in nitrified water samples from chloraminated systems (Sarker 2012). This could be due to two reasons. First is the presence of microbes that are not capable to produce cSMP under given conditions, such as high organic carbon concentration, which may inhibit AOM. The energy derived from reducing carbon dioxide to cellular carbon poses a

greater competitive disadvantage to nitrifying microbes than to heterotrophs (Rittmann, Bruce E & Manem 1992). Second, the presence of NOM or other agents (silver like substances) in the water may inhibit or enhance the reactions or production/action of cSMP. Therefore, it is important to understand how different levels of DOC affect cSMP in severely nitrifying samples.

This chapter reports the results from reactors fed with water produced at Orchard Hills Water Treatment Plant, Sydney, Australia, over a period of one year. Throughout the different seasons, the organic matter concentration varied in the source water, and hence in the treated water. The impact of such a change on the amount of cSMP in severely nitrified water was analysed. As there is no direct measurement method, the impact of cSMP on chloramine decay or nitrogenous species (ammonia, nitrite, and nitrate) in filtered samples was compared between nitrified and feed samples incubated under the same initial conditions. To confirm that the effect was actually caused by cSMP but NOM, cSMP was isolated and reintroduced into water with low NOM. Isolation was achieved by size fractionation with 30, 50, and 100 kDa centrifugal ultrafiltration membranes. If accelerated chloramine decay associated with faster nitrite oxidation, higher inorganic nitrogen loss should be occurred. In addition, the protein concentration was measured in the isolated fraction to confirm its presence. The mechanisms were confirmed by conducting stoichiometric calculations in the samples without NOM.

4.2 Materials and methods

4.2.1 Operation of lab scale reactor systems

The lab-scale reactor sets were operated as described in Chapter 3, section 3.3 in a room with a temperature of 20–23 °C. At any stage, two parallel reactor sets were operated; and after those had been fully examined, the next two reactor sets were operated. Each reactor set was fed with different DOC contained water (0-1, 2-3, 4-5 and 7-8 mg-C.L^{-1}) as the feed water. Each lab-scale reactor system was continuously operated approximately 72 d during the experimental period.

4.2.2 Experimental design

The cSMP is often present in severely nitrified water samples, and can pass through a 0.22 µm membrane (Krishna, Sathasivan & Sarker 2012). In samples containing cSMP, chloramine decay is associated with the increased reduction rates of nitrite and TAN than feed water that has not been subjected to nitrification (Sathasivan & Bal Krishna 2012). To quantify the cSMP concentration, various samples were prepared and the impact of cSMP on total chlorine, nitrite, NOx, and TAN were compared between filtered nitrified and feed water samples collected from the reactor sets fed with different DOC levels.

The subsamples were prepared from R-5 (severely nitrified) and feed (non-nitrified) samples by filtering some of the sample through 0.22 µm sterilised 33 mm syringe driven filter units [housing limit 150 psi (10 bars), non-pyrogenic, Millipore Express® PES Membrane, MILLEX®-GP, MILLIPORE IRELAND Ltd]. The resulting samples were placed in 500 mL PET bottles and labelled as R-5 filtered, R-5 unfiltered, Feed filtered, and Feed unfiltered.

The initial chemical compositions, including residual chloramine, nitrite, and pH, were adjusted in each sample using the respective stock chemical solutions. To achieve the expected chloramine concentration in the water sample, ammonia was added (maintaining Cl- to- TAN mass ratio of 4:1) prior to chlorine addition. The nitrite level was adjusted depending on the concentration in R-5 at the time of sampling and the pH was adjusted to 7.9–8.1. The samples were incubated at 20 °C in a water bath.

For each sample, the total chlorine was monitored and the decay coefficient was estimated using exponential regression in Excel (Microsoft®). When chlorine was added to the sample, the same dose was added to Milli-Q water, and the chlorine concentration was measured to ensure that the correct dose was applied. All experiments were conducted in duplicate.

To separate the role of cSMP from that of NOM, size fractionation (Section 4.2.2) was conducted. As cSMP is suspected to be proteins, the protein concentrations of some of the solutions were measured (Filik & Stone 2009).

Collect severely nitrified bulk water (2L) from the R-5 reactor
fed with 0-1 mg-C.L^{-1} DOC contained feed water

→ R-5 Filtered sample

Filter through 0.22 µm membrane to eliminate microbes

Ultracentrifugation for protein separation

100 kDa retentate ←   50 kDa retentate →   30 kDa retentate →

100 kDa filtrate ←   50 kDa filtrate →   30 kDa filtrate →

100 kDa   50 kDa   30 kDa
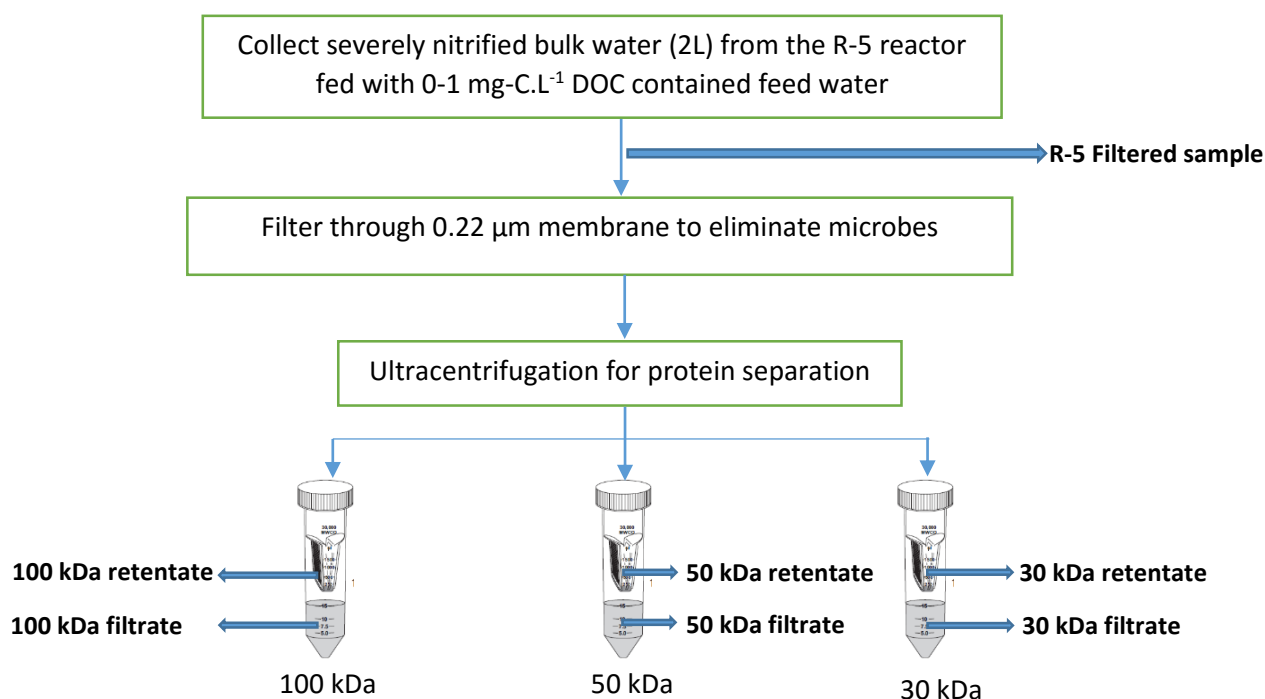
Figure 4.1: Detailed experimental plan for preparation of ultra-centrifugal samples

4.2.3 Size fractionation of cSMP

To elucidate the role of cSMP in accelerating chloramine decay, centrifugal ultra-filtration was conducted to separate the cSMP containing fraction from other organic compounds in the nitrified water samples (Fig. 4.2). The reactor set that was fed with water containing 0.1 mg-C.L^{-1} of DOC was used to collect the nominated samples. As most NOM has a maximum MW of < 10 kDa, the use of > 30 kDa MW ultra-centrifugal filters clearly separated NOM. Three 2 L aliquots of 0.22 µm filtered samples were filtered through centrifugal filters (x4000 g for 6 min) of 100, 50, and 30 kDa MW. The filtrates (~1.9 L) and retentate (~10 mL) were collected, and the retentate from each filter was mixed with 90 mL of 0.22 µm filtered feed water (DOC < 0.1 mg-C.L^{-1}) and named 100, 50 or 30 kDa retentate samples. From the centrifugal filtrates, 100, 50, or 30 kDa filtered samples were also collected. Similar to Section 4.2.1, the initial conditions (chloramine, Cl/TAN, nitrite, and pH) were adjusted in all samples, including R-5 filtered and feed filtered samples, from the same reactor. cSMP was quantified by comparing time series profiles of the respective sample with the low DOC containing feed.

4.2.4 Protein quantification

In this experiment, EZQ (Invitrogen) Protein Assay from Invitrogen was used to determine the protein concentration in the filtered nitrified water sample. The retentate (~10 mL) from filtering 2 L of the sample through 0.22 µm followed by 30 kDa was used for protein measurement. The proteins were further concentrated by lyophilisation. In this procedure, proteins were concentrated through freeze-drying, while they maintained their tertiary structure. The sample then underwent a highly sensitive EZQ fluorescent assay process to determine protein concentration. Known bovine serum albumin (BSA) concentrations were used to prepare the standard curve in a 2% sodium dodecyl sulphate (SDS) buffer. The fixing step involved incubation for 5 min in 100% methanol under constant shaking at room temperature. Incubation was conducted with 50 mL of an EZQ protein quantitation reagent (Invitrogen Cat # R33200, component A) for 30 mins under constant rocking (50 rpm). After incubation, the sample was washed with a solution containing a mixture of 10% methanol and 7% acetic acid solution three times, with an interval of 20 s between each wash. Fluorescence was detected by a FUJI LAS-4000 scanner using SYBR green fluorescence settings. Image analysis and quantification were conducted using Multi Gauge software. The standard curve had an R^2 value of 0.9967.

4.2.5 Chloramine decay mechanisms

Eq. 4.1 and 4.2 were used to determine chloramine losses due to auto-decomposition and nitrite oxidation, respectively. During TAN loss by auto-decomposition, nitrogen in TAN converts into N_2 gas. According to Eq. 4.1, 1 mol of N_2 gas (28.0 g of nitrogen) is lost due to the decay of 3 mol of chloramine (3×71.0 g of Cl_2) due to auto-decomposition. Therefore, approximately 7.50 mg of chlorine is lost when 1 mg of TAN is lost.

According to Eq. 4.2, 1 mol of nitrite (14.0 g as nitrogen) will result in the loss of 1 mol of chloramine (71.0 g as Cl_2) by nitrite oxidation. Hence, 5 mg of chloramine would be lost by oxidizing 1 mg of nitrite.

4.3 Results and discussion

4.3.1 Performance of the continuous flow reactor sets during sample collection

The reactors were operated at different times of the year with varying DOC levels as dictated by variations in the source water and treatment processes (Table A-1). There were significant differences in the total chlorine residuals and associated changes in nitrogenous compounds between R-5 and the feed water samples in each reactor set (Table 4.1). These changes were due to the increase of nitrifying bacterial activity when water moved from the first (R-1) to the last reactor (R-5) in each reactor set. The pH simultaneously decreased from 8±0.1 to 7.6–7.7, another indicator of nitrifying bacterial activity (Tuovinen et al. 1980). As noted previously, the maximum cSMP production/presence was associated with severe nitrification, such as that in R-5, where the highest nitrification was observed (Krishna, Sathasivan & Sarker 2012). Therefore, the presence of cSMP was assessed in the samples from the final reactor (R-5).

The DOC level did not change greatly between the first and last reactors of each set (Table 4.1). If the cSMP concentration was high enough, the DOC level would have increased when the water travelled from R-1 to R-5. Therefore, cSMP must be present at a low concentration which may not be measured by assessing DOC, therefore, other means of measurement are required.

Table 4.1: Parameters of the collected water samples before the adjustment for batch experiments

Parameters	Reactor set with 7-8 mg-C.L^{-1} DOC on day 50		Reactor set with 4-5 mg-C.L^{-1} DOC on day 50		Reactor set with 2-3 mg-C.L^{-1} DOC on day 50		Reactor set with 0-1 mg-C.L^{-1} DOC on day 50	
	Feed	R-5	Feed	R-5	Feed	R-5	Feed	R-5
Total Chloramine (mg-l$_2$.L^{-1})	2.0±0.03	0.0±0.03	1.92±0.03	0.0±0.03	1.95±0.03	0.0±0.03	2.2±0.03	0.0±0.03
TAN (mg.L^{-1})	0.500±0.005	0.018±0.004	0.485±0.006	0.012±0.002	0.522±0.005	0.025±0.005	0.522±0.004	0.031±0.006
Nitrite (mg-N.L^{-1})	0.0±0.003	0.410±0.003	0.0±0.003	0.320±0.003	0.0±0.003	0.320±0.003	0.0±0.003	0.300±0.003
Ph	7.98-8.02	7.69-7.72	7.92-7.95	7.61-7.65	8.01-8.05	7.62-7.66	7.98-8.01	7.66-7.68
DOC (mg-C.L^{-1})	7.23±0.13	7.28±0.13	4.26±0.08	4.29±0.08	2.50±0.10	2.58±0.10	0.23±0.07	0.29±0.07

4.3.2 The impact of DOC on the concentration of cSMP in reactors

The results of the batch experiments showed two distinct chloramine decay behaviours. The samples collected from the reactors fed with water that contained 0–5 mg-C.L^{-1} DOC behaved similarly, whereas those containing 7–8 mg-C.L^{-1} DOC behaved differently; these were denoted as Type I and II behaviours, respectively.

Type I behaviour observed in reactor sets operated with 0–5 mg-C.L^{-1}

The profiles of total chlorine, nitrite, TAN, and NOx (Fig. 4.2) in water samples collected from the severely nitrified reactor (R-5) that was operated with feed water containing 4–5 mg-C.L^{-1} DOC exhibits Type I behaviour. To determine if cSMP were present, the same initial conditions were applied to all samples, and the behaviours were compared as described in Section 4.2.1.

Differences between the filtered and unfiltered samples can be attributed to the microbes or filterable products present in unfiltered samples. The difference is minimal in the feed water, and the profiles obtained from the feed water sample explain the absence of cSMP, i.e., the lower TAN loss (0.026 ± 0.03 mg.L^{-1}), slower conversion of nitrite to nitrate, and considerably slower decay of chloramine (Figs 4.2a-c). The NOx profile (constancy) also explains the absence of nitrification in the feed water sample. This observation is similar to that observed in mildly nitrifying samples by Sathasivan, Fisher and Tam (2008).

In contrast, the difference between the filtered and unfiltered samples of R-5 clearly indicated the presence of nitrifier activity (increasing NOx-N) in the unfiltered sample. In the R-5 filtered sample, there was no nitrifier activity (constant NOx-N).

(a)

(b)

(c)

(d)

- Feed Unfiltered   - R-5 Unfiltered   - Feed Filtered   - R-5 Filtered
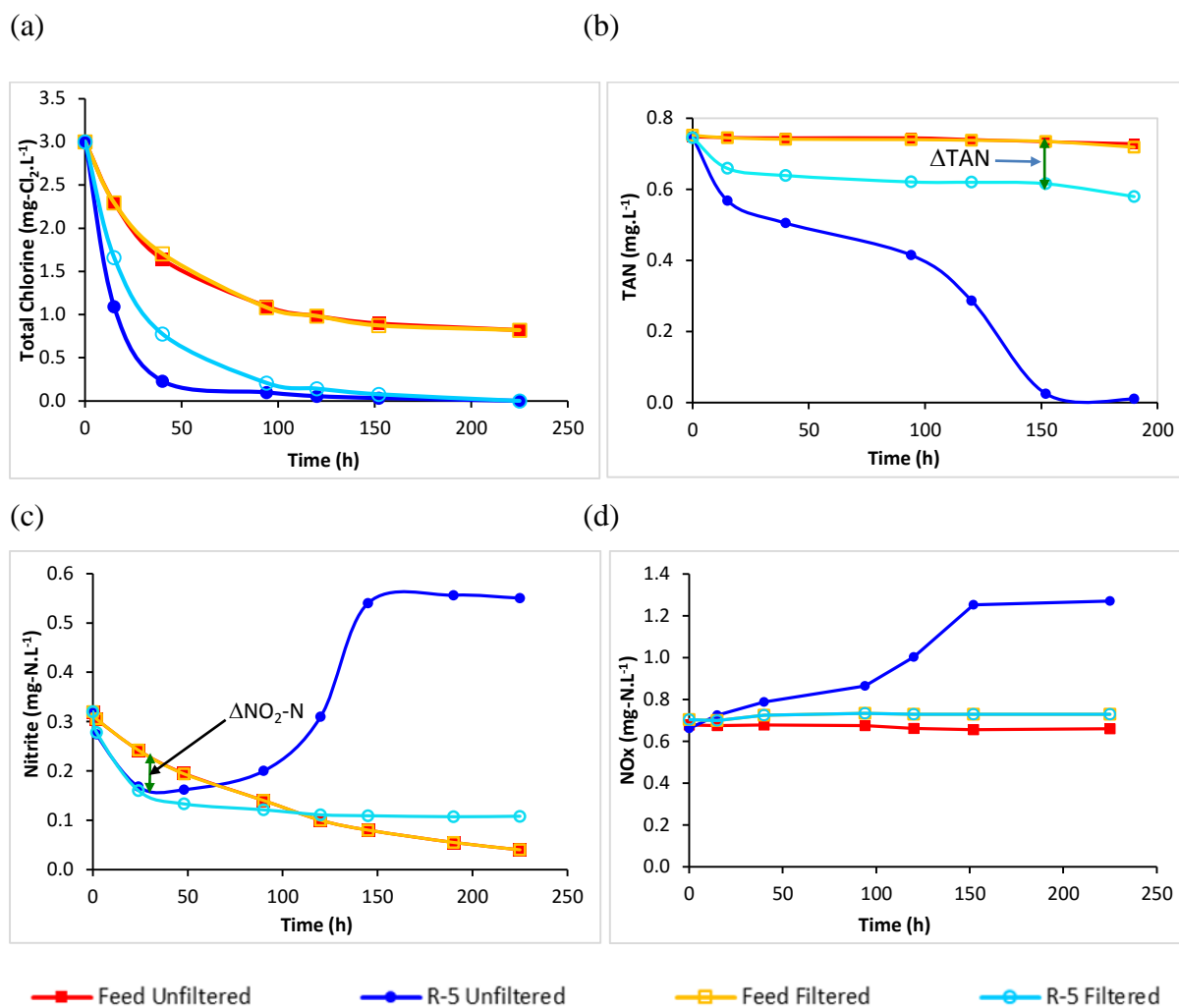
Figure 4.2: Decay profiles in the unfiltered and filtered bulk water samples of feed (Initial nitrite level adjusted to a similar level as R-5 water) and R-5 collected from reactor set fed with 4-5 mg-C.L^{-1} DOC containing feed water (an example of Type I behaviour).

(a): Total chlorine, (b) TAN, (c) Nitrite and (d) NO$_x$. ΔTAN and ΔNO$_2$-N measure the impact of SMP accelerating auto-decomposition and nitrite oxidation.

The differences in the behaviour of filtered samples from the feed and R-5 can be attributed to the presence of cSMP, as there was no microbial activity (Krishna, Sathasivan & Sarker 2012). The amount and rate of TAN decrease and nitrite conversion were higher in the R-5 filtered sample than that in the feed water (Figs 4.2b-c). This was associated with an increased chloramine decay. During the initial 30 h, these changes were more noticeable. This suggests that cSMP are present in the R-5 filtered sample (Sathasivan & Bal Krishna 2012). After the

chloramine level had decreased below 0.40 mg-Cl_2.L^{-1}, the nitrite level remained the same as the level of chloramine was insufficient to oxidise nitrite. A gradual decrease in the nitrite level was observed in feed water samples until the end of the incubation period, at which there was a higher residual chloramine concentration (Figs 4.2a and c).

Table 4.2: The impact of cSMP in filtered severely nitrified samples ΔTAN and ΔNO2-N in Type I and Type II samples

Impact of cSMP in 0.2 μm filtered R-5	DOC (mg-C.L^{-1})			
	Type I			Type II
	0-1	2-3	4-5	7-8
ΔNO$_2$-N (mg.L^{-1})	0.060±0.003	0.068±0.003	0.085±0.003	0.00±0.003
ΔTAN (mg.L^{-1})	0.242±0.006	0.124±0.005	0.129±0.002	0.058±0.004

The change in the total inorganic nitrogen (TIN) of the R-5 filtered sample was 0.129 mg.L^{-1}. This is the same as the change in TAN (ΔTAN) from the reactions associated with cSMP as there is no net NOx gain in the absence of microbes (Fig. 4.2d). Similarly, the further decrease in nitrite (ΔNO$_2$-N) of the filtered R-5 sample is due to cSMP. The ΔTAN (0.129 ± 0.03 mg.L^{-1}) and ΔNO$_2$-N (0.085 mg.L^{-1}) explain the impact of cSMP on auto-decomposition and nitrite oxidation, respectively, over and above the feed water samples (Krishna, Sathasivan & Sarker 2012). The impact of cSMP, therefore, can be measured using the maximum ΔTAN and ΔNO$_2$-N values, which are used as a surrogate. Similar values were calculated for the samples collected from reactors operated with feed water that contained other DOC values between 0–6 mg.L^{-1}, and are presented in Table 4.2.

Type II behaviour observed in 7–8 mg-C.L^{-1} DOC containing reactor set

The same initial conditions as the Type I batch experiments were maintained in all samples (Fig. 4.3). In Type II, the R-5 filtered sample behaved similarly to the Type I R-5 sample with few major differences (Fig. 4.3). An absence of ΔNO$_2$-N and less ΔTAN (0.058 mg.L^{-1}) than the Type I sample were observed in the Type II R-5 filtered sample. In these profiles, cSMP is

present at a lower level. However, the chloramine decay characteristics of the Type II R-5 unfiltered sample were similar to those of the Type I R-5 unfiltered sample.

(a)

(b)

(c)

(d)
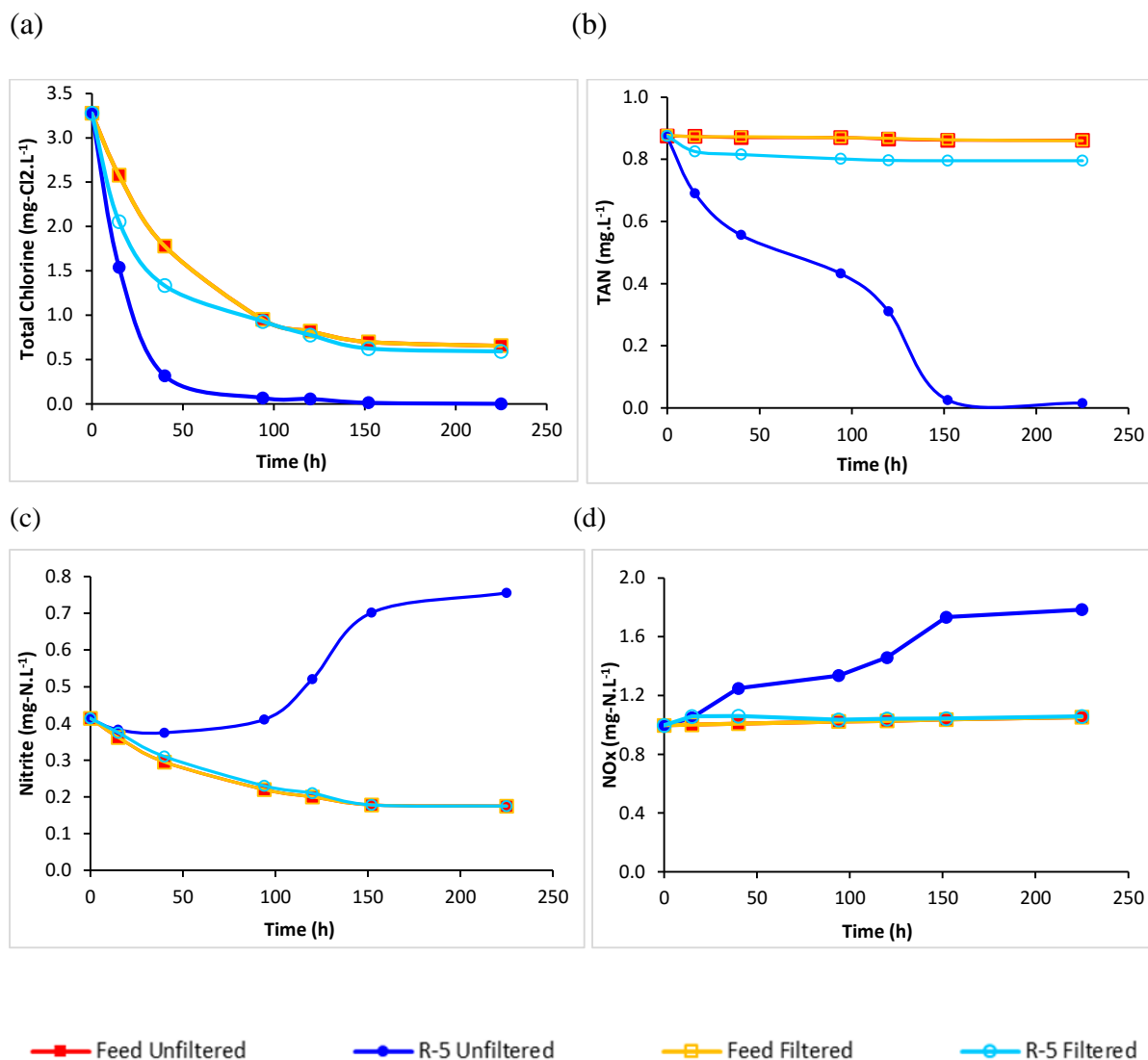
Figure 4.3: Decay profiles in the unfiltered and filtered bulk water samples from feed and R-5 collected from reactor set fed with 7-8 mg-C.L^{-1} DOC containing water (Type II behaviour). (a) Total chlorine, (b) TAN, (c) Nitrite and (d) NO$_x$. Feed water had nitrite level adjusted to nitrite levels in R-5.

Summary of the impact of DOC on the production of cSMP

There was a decrease in the impact of the cSMP (ΔNO_2-N and ΔTAN) when the feed water DOC level increased (Table 4.2). The sample from the reactor that was fed with the lowest DOC-containing water was the most affected by cSMP (Table 4.2). For water containing the usual range of DOC from many utilities, which is often between 3 and 5 mg-C.L^{-1}, there was

no significant difference in the impact of cSMP. In the samples from the reactor fed with the highest DOC (7–8 mg-C.L^{-1}), such as those in Type II, cSMP did not accelerate nitrite oxidation (ΔNO_2-N) and it had less of an effect on ΔTAN. The lower effect of cSMP in high DOC reactor samples could be because of two possible reasons; the presence of inhibitors of cSMP activity or that of microbes responsible for producing cSMP. DOC itself may not act as an inhibitor to cSMP activity. Since unusually high DOC was registered following a significant rain event, it is hypothesized that inorganic inhibitors could be present in the water sample.

Although the DOC values of feed water were noticeably different, impacts by other trace elements/substances that are naturally present in collected water could not be ruled out, as the water was collected at different times of the year. Furthermore, the feed water containing 0–1 mg-C.L^{-1} was prepared by sending the water through biologically activated carbon (BAC), which can remove other trace elements in addition to NOM (or biodegradable dissolved organic carbon) (Korotta-Gamage & Sathasivan 2017). To relate the impact to production, the impact of other agents (such as NOM, nitrite) should be eliminated, or the cSMP should be isolated and the behaviour should be characterised. This is discussed further in Section 4.3.3.

4.3.3 Isolation of cSMP and protein measurement

If cSMP were present in a water sample, an accelerated chloramine decay (associated with accelerated auto-decomposition and/or nitrite oxidation) should be observed (Krishna, Sathasivan & Sarker 2012). Therefore, the introduction of cSMP into the chemical parameter-adjusted BAC-treated feed water (< 0.1 mg-C.L^{-1} DOC) should also decay chloramine. Consequently, if cSMP was successfully separated through centrifugal filters, a clear higher decay rate associated with additional losses of TAN (ΔTAN) and nitrite (ΔNO_2-N) should be observed in retentate water samples, as opposed to the filtrate samples. This explanation corresponded with the decay results observed from the 30 kDa centrifugal filter units (Fig. 4.4b, d and f), but not from either the 100 or 50 kDa filter units (Fig. 4.4a, c and e).

The results for the samples prepared from 30 kDa retentate show that the impacts of cSMP in both retentate (concentration factor of 20) and original samples (R-5) were similar (Fig.4.4b). This could be due to the low recovery (5%) of proteins during centrifugal filtration as they may have been attached to filter papers. Centrifugal filtration uses 10 mL each time, and the filters should be replaced every 30 mL. Even though the 100 and 50 kDa retentate samples exhibited

a low decay rate, as observed in the feed filtered samples, the 100 and 50 kDa filtrate samples exhibited higher decay rates close to those of the R-5 filtered sample (Fig. 4.4a).
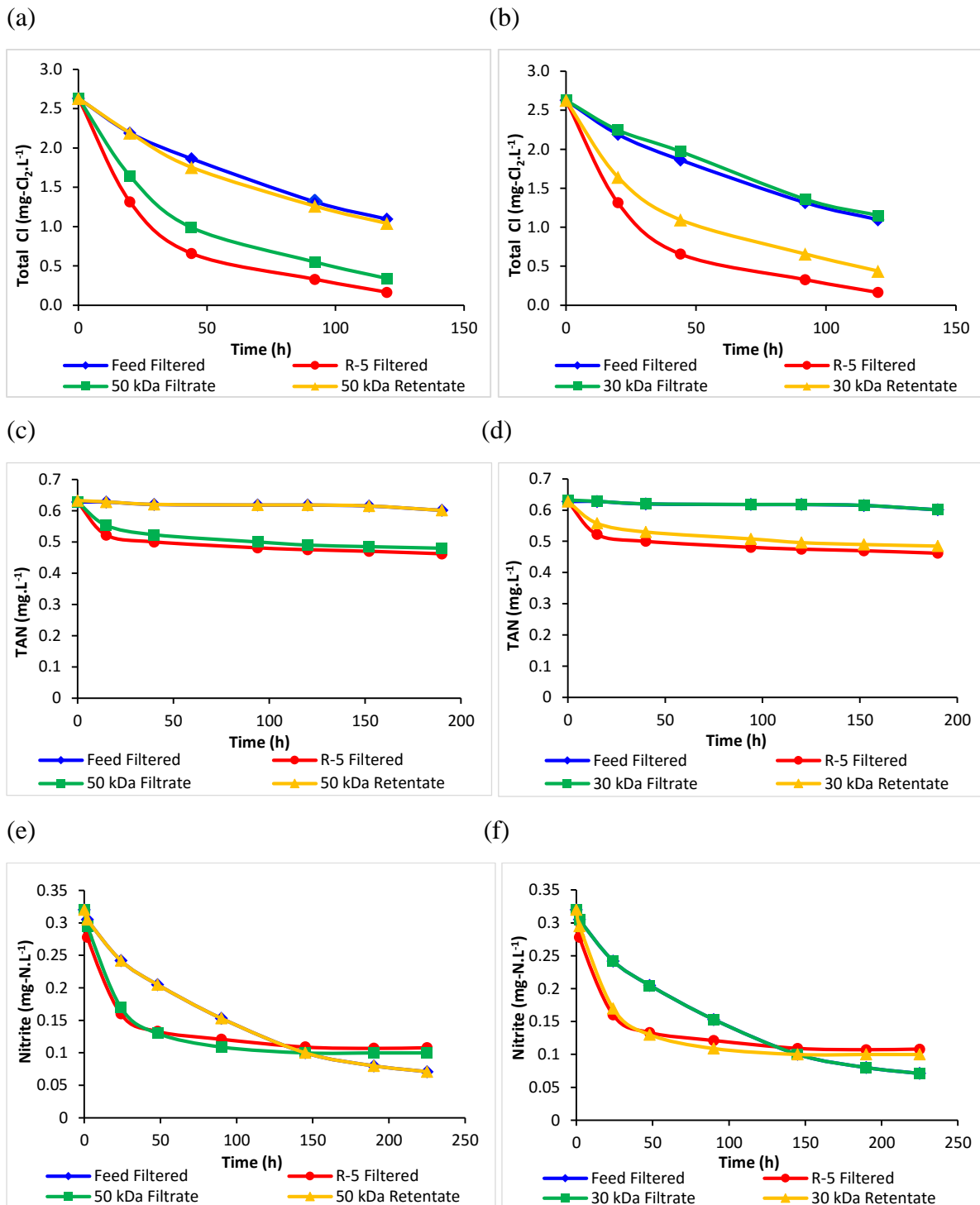
Figure 4.4: Decay profiles of water samples. 5 (a) and (b) Total chlorine, 5 (c) and (d) TAN and 5 (e) and (f) Nitrite. 50 kDa and 30 kDa represent the samples tested for respective ultracentrifugation. 100 kDa ultra centrifugal samples (data not shown) showed the similar results as 50 kDa samples.

Similarly, the TAN and nitrite profiles indicated that there was no cSMP in the 100 or 50 kDa retentate samples (Fig 4.4c and e). TAN loss was comparatively insignificant from the beginning, and there was also no observable rapid decay of nitrite. Nevertheless, the 100 and 50 kDa filtrate samples confirmed the presence of cSMP due to the additional loss of TAN and faster loss of nitrite.

This observation is probably due to the cSMP passing through the 100 and 50 kDa filters. This demonstrates that cSMP are smaller than the pore sizes of 100 and 50 kDa MW membranes. The protein concentration in the 30 kDa retentate results was 0.05 mg.mL^{-1}, possibly indicating a very low cSMP concentration. These results indicated that cSMP could contain proteins and have a molecular size of 30–50 kDa, and it behaves in a similar manner to that observed in severely nitrifying water.

4.3.4 Chloramine decay mechanisms by cSMP

The chloramine demands arising from both auto-decomposition (Eq. 4.1) and nitrite oxidation (Eq. 4.2) were calculated as per Section 4.2.4, and compared in Fig. 4.5a for the R-5 30 kDa retentate sample with cSMP. The results for all samples examined at 120 h are presented in Table 4.3. In the samples containing cSMP, such as R-5 filtered, 100/50 kDa filtrate, and 30 kDa retentate, the chloramine demand can be explained by auto-decomposition and nitrite oxidation. The decay of chloramine was not accelerated in the 30 kDa filtrate samples (Fig. 4.5b) as there was no cSMP present. The nitrite oxidation in samples that did not contain cSMP was slower than that in the samples that contained cSMP. This is because the cSMP-containing samples fully oxidized nitrite within the first 45 h, while the samples that did not contain cSMP could not achieve the maximum oxidation, even within 120 h of incubation time.

Table 4.3: Summary of the nitrogen mass balance and chloramine decay mechanisms in the first 120 h of incubation time

Parameters		Feed water[1]	Nitrified water[1]					
		0.22 μm filtration	0.22 μm filtration	0.22μm followed by 100/50 kDa filtration		0.22μm followed by 30 kDa filtration		
		Filtrate	Filtrate	Filtrate	Retentate + Feed water	Filtrate	Retentate + Feed water
TAN loss (mg.L^{-1})		0.010±0.024	0.153±0.022	0.139±0.022	0.014±0.025	0.014±0.025	0.132±0.021
Nitrite loss (mg-N.L^{-1})		0.196±0.008	0.206±0.008	0.216±0.008	0.196±0.008	0.196±0.008	0.216±0.008
Chloramine demand (mg-Cl$_2$.L^{-1})	**Auto-decomposition**	0.07±0.17	1.15±0.17	1.04±0.17	0.10±0.18	0.10±0.18	0.99±0.17
	Nitrite oxidation	0.98±0.04	1.03±0.04	1.08±0.04	0.98±0.04	0.98±0.04	1.08±0.04
	Nitrite oxidation and auto-decomposition	1.05±0.21	2.18±0.21	2.12±0.21	1.08±0.22	1.08±0.22	2.07±0.21
	Measured	1.53±0.10	2.47±0.10	2.29±0.10	1.59±0.10	1.47±0.10	2.19±0.10
	Other reactions	0.48±0.31	0.19±0.31	0.17±0.31	0.51±0.32	0.39±0.32	0.12±0.31

[1]The feed water and nitrified water contained a DOC of 0.23 and 0.29 mg-C.L^{-1} respectively

(a)
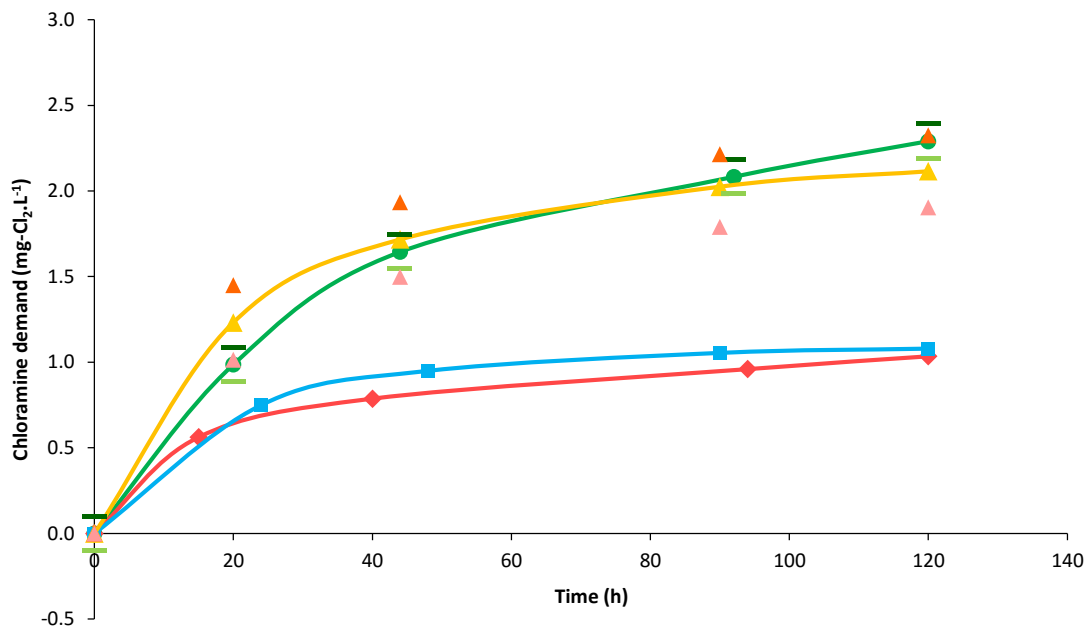
(b)
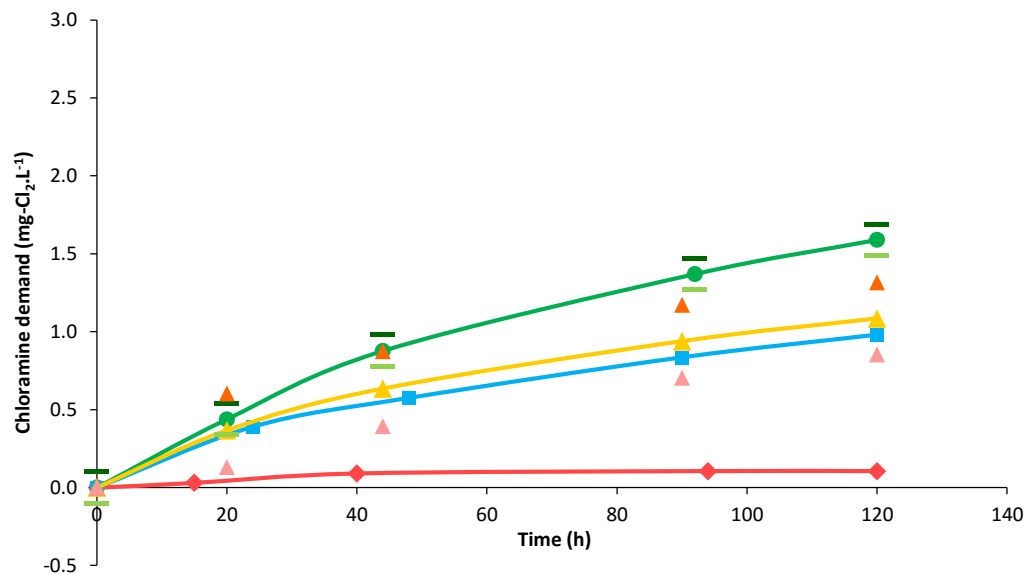
Figure 4.5: Chloramine demand comparison by Auto decomposition and nitrite oxidation. 4.5a: 30 kDa retentate (representation of cSMP presence). 4.5b: 30 kDa filtrate.

As can be expected, the unfiltered samples had a higher chloramine demand than the filtered samples, i.e., the cellular products and microbial metabolism (such as nitrite production) in the nitrifying samples could be responsible for such additional decay. Further investigation is required to elucidate further mechanisms.

4.3.5 Implications for further research and the chloraminated water supply systems operation

Table 4.4 shows the relative contribution of various factors to accelerating chloramine decay within the first 40 h of the re-chlorination of nitrifying water collected from reactors fed with 0-8 mg-C.L^{-1} DOC. It is evident that cSMP play a critical role in 0-5 mg-C.L^{-1} fed reactors; 21–39% of all chloramine demand was induced by cSMP. In these samples, about 40-66% was explainable by reactions associated with auto-decomposition and oxidation of NO$_2$ and NOM already present in the water at the time of dosing and about 13-21% by filterable matter (>0.2 µm). In the samples from 7-8 mg-C.L^{-1} fed reactors, the role of cSMP is subdued at 15%, but that of filterable matter accounted for 34%. All possible mechanisms for the chloramine decay by the filterable organic matter are explored below for samples from both 4-5 and 7-8 mg-C.L^{-1} fed reactors (Figs 4.2 & 4.3), where the measured additional chloramine demand exerted by filterable matter is 0.55 and 1.02 mg-Cl$_2$.L^{-1}.

Oxidation of nitrite produced from nitrification by chloramine is one possible mechanism. Amounts of nitrite oxidised in samples from 4-5 and 7-8 mg-C.L^{-1} fed reactors are 0.10±0.08 and 0.19±0.12 mg-N.L^{-1}, respectively (Table 4.5). These could be utilised to calculate the chloramine demand, if nitrite oxidising bacteria were not present. However, nitrite oxidising microbial community, such as *Nitrospira spp.*, is regularly found in nitrifying samples of chloraminated systems (Krishna, Sathasivan & Ginige 2013; Nagymáté, Homonnay & Márialigeti 2016; Regan, John M, Harrington & Noguera 2002).

Proteins associated with biomass or that are released by microbes in the biomass could accelerate the chloramine decay. If there are chloramine decaying proteins in the bulk water, the biomass can have the same type of proteins but in an insoluble form (Rittmann, Bruce E 2018). Proteins could be released by microbes as a response to the stress (chloramination). (Wang, Z-P & Zhang 2010) reported that stress, such as low pH, induces production of protein-like substances and that the stress rather than the species determine the release of SMP such as

proteins. Chlorination can induce the antibacterial resistance genes in microbes which may also be the reason for the observation of additional impact of SMP (protein) in the bulk water (Shi, P et al. 2013). Additional nitrogen loss in samples from 4-5 and 7-8 mg-C.L^{-1} reactors have high error (0.12±0.10 and 0.00±0.14, respectively) (Table 4.5) and some inorganic nitrogen could be utilised for assimilation. Therefore, additional chloramine loss from nitrogen loss cannot be completely elucidated, but needs future experiments to elucidate such mechanisms.

Table 4.4: The relative contribution of different factors for chloramine demand in the R-5 severely nitrified reactors using reactor sets maintained with different DOC levels within first 40 h of the incubation period

	Reactor set with 0-1 mg-C.L^{-1} DOC level		Reactor set with 2-3 mg-C.L^{-1} DOC level		Reactor set with 4-5 mg-C.L^{-1} DOC level		Reactor set with 7-8 mg-C.L^{-1} DOC level	
	Chloramine demand (mg-Cl$_2$.L^{-1})	Contribution (%)	Chloramine demand (mg-Cl$_2$.L^{-1})	Contribution (%)	Chloramine demand (mg-Cl$_2$.L^{-1})	Contribution (%)	Chloramine demand (mg-Cl$_2$.L^{-1})	Contribution (%)
Oxidation of residual nitrite, auto-decomposition and reaction with NOM (from feed filtered samples)	1.10	40	1.94	66	1.30	47	1.50	51
Reactions expedited by cSMP (difference between R-5 filtered and feed filtered samples)	1.08	39	0.60	21	0.93	33	0.44	15
Other reactions (difference between unfiltered and filtered R-5 samples)	0.57	21	0.39	13	0.55	20	1.02	34
Total	2.75	100	2.93	100	2.78	100	2.96	100

Finally, heterotrophic bacteria could play an additional role to accelerate chloramine decay by inducing cSMP production or other means such as direct utilisation. Heterotrophic microbes with versatile ability to degrade various compounds are found in chloraminated systems (Krishna, Sathasivan & Ginige 2013; Nagymáté, Homonnay & Márialigeti 2016) and a complete decay of all chloramine (1.5 mg-Cl$_2$.L^{-1}) has been noted without nitrification in a laboratory scale reactor (Herath, Sathasivan & Lam 2015).

Table 4.5: Summary of nitrogen mass balance in the unfiltered as opposed to filtered R-5 (severely nitrifying) sample within the first 40 h

Nitrogen species	**Reactor set with 4-5 mg-C.L^{-1} DOC level**	**Reactor set with 7-8 mg-C.L^{-1} DOC level**
NO$_x$ production from nitrification (mg-N.L^{-1})	0.13±0.07	0.26±0.11
NO$_2$ increase (mg-N.L^{-1})	0.02±0.01	0.07±0.01
Oxidised NO$_2$ (mg-N.L^{-1})	0.10±0.08	0.19±0.12
Nitrogen loss (change in total inorganic nitrogen) (mg.L^{-1})	0.12±0.10	0.00±0.14

The results that almost all chloramine is lost within 40 hr of re-chloramination in all tested samples suggest there is not much benefit in removing organic carbon if nitrification is allowed to take place (Table 4.4). The removal of organic carbon, however, can produce benefits by minimising chemical chloramine decay caused by reactive NOM (Vikesland, Ozekin & Valentine 1998), and possibly microbial chloramine loss mediated by heterotrophic bacteria (Herath, Sathasivan & Lam 2015) thus postponing the onset of nitrification (Sathasivan, Fisher & Tam 2008). Eventually, if chloramine residuals decrease below the biostable residual concentration (Sathasivan, Fisher & Tam 2008), higher nitrification rates associated with heterotrophic microbial growth (Krishna, Sathasivan & Ginige 2013) and production/impacts of protein can be expected, as observed in the reactors fed with low DOC-containing samples. Additional chlorine needs to be added to overcome the impact of protein if breakpoint chlorination is carried out (Krishna, Sathasivan & Kastl 2014). If DOC can be reduced below 2.5 mg-C.L^{-1}, the benefits from chlorine may outweigh those of chloramine, especially if the

water contains a low bromide concentration. Since DOC level is less than 2.5 mg-C/L, chlorine is more stable and form less THM in the water.

4.4. Conclusion

Four lab-scale reactor sets were operated using water collected from a water treatment plant during different times of the year to understand the impact of DOC level on the production of chloramine-decaying SMP (cSMP) under severely nitrifying conditions. The feed water contained varying DOC concentrations. Chloramine decay experiments were conducted by separating microbial and chemical agents after adjusting the initial levels of chloramine, ammonia, and nitrite so that they were the same. The additional acceleration of chloramine decay by auto-decomposition and nitrite oxidation beyond that of feed water samples was used to understand the impact of cSMP. To confirm that the observed effect was caused by cSMP and not NOM, cSMP were separated by 30, 50, and 100 kDa centrifugal filters and tested with water containing less than 0.1 mg-C.L^{-1} of DOC. The conclusions from these two experiments are as follows:

- The impact of cSMP on severely nitrifying samples obtained from each reactor set generally decreased as the DOC of the feed water increased.

- The impact of cSMP was minimal in severely nitrifying samples from the reactor fed with the highest DOC concentration (7–8 mg-C.L^{-1}). As this DOC level is unlikely in a typical water supply system, it is not practically significant.

- cSMP is larger than 30 kDa but smaller than 50 kDa, and contains proteins.

- All chemical chloramine decay in cSMP-containing samples can be explained by nitrite oxidation and auto-decomposition.

- When severely nitrifying water with a DOC of 0-5 mg-C.L^{-1} is rechloraminated, approximately 21–39% of the chloramine is required by cSMP within the first 40 h. The filterable material accounted for 13-21%.

Chapter 5

Bacterial community composition on the laboratory scale reactor systems operated by feeding chloraminated water containing different dissolved organic carbon (DOC) levels

5.1 Introduction

Chloramine plays a significant role in drinking water disinfection due to the formation of less disinfection by-products (DBPs) and sustaining long lasting residuals in the distribution system. There are three different forms of inorganic chloramine (monochloramine, dichloramine and trichloramine) and the most desirable form of chloramine in water distribution system is monochloramine. However, monochloramine is naturally unstable as it tends to auto-decompose by a complex set of reactions (Jafvert & Valentine 1992). Chloramine residual is required to be as stable as possible to be in line to meet disinfectant residuals and to minimize DBPs formation.

Conversely, in addition to nitrification, chloramine decaying protein-CDP (previously known as cSMP) accelerates chloramine decay much faster by acting as a catalyst for auto

decomposition and nitrite oxidation (Heath et al 2018). Nitrification is a two-step biological process whereby ammonia is converted to nitrite by ammonia oxidising microbes (AOM) and then the nitrite is further oxidised in to nitrate by nitrite oxidising bacteria (NOB). The impact of CDP can be observed notably in severely nitrifying conditions (nitrite > 0.01 mg-N.L^{-1}). Exceptional circumstances have been reported by Sarkar (2012) as that the CDP activity is not always amply demonstrated in nitrified water samples obtained from chloraminated systems.

Experiments conducted by Herath, Torres and Sathasivan (2018) had confirmed this observation as the nitrified lab scale reactor set comprised of high level of dissolved organic carbon-DOC (>7 mg-C.L^{-1}) did not express CDP impact. Only the nitrified water that contained DOC levels of 0-5 mg-C.L^{-1} have shown CDP activity. Therefore, DOC might play an important role on controlling the microbes responsible for CDP production or inhibiting the expression of CDP impact. As nitrifying micro-organisms are autotrophs, they do not depend on organic carbon sources like natural organic matter (NOM). The survival of heterotrophic micro-organisms depends completely on carbon availability. Therefore, heterotrophs could play an important role in inhibiting CDP presence or/and activity depending on the carbon availability in reactors. However, Herath, Sathasivan and Lam (2015) reported that as heterotrophs could also decay chloramine to the same degree as the nitrifying micro-organisms.

Identification of micro-organisms present in different DOC containing reactors could help to expose the mystery behind accelerated chloramine decay due to CDP, as well as leading for a way to control the impact of CDP activity on chloramine decay. Several experiments have revealed the community composition in chloraminated drinking water distribution system. In both chloraminated and chlorinated water, the most predominant bacterial class was Alphaproteobacteria (Williams et al. (2004) whereas the Betaproteobacteria was more sensitive to disinfection. The dominant families under chloramination were Methylophilaceae, Methylococcaceae, and Pseudomonadaceae (Hwang et al. (2012). Krishna, Sathasivan and Ginige (2013) reported the bacterial community changes with decaying chloramine residuals in a lab-scale system operated with 2.80-3.0 mg-C.L^{-1} DOC level. According to the results, the dominant bacterial classes were Solibacteres, Nitrospira, Sphingobacteria, and Betaproteobacteria with low chloramine residuals (< 0.65 mg.L^{-1}) and high nitrification activity. However, when higher chloramine residuals (1.60-2.18 mg.L^{-1}) were maintained, classes Actinobacteria and Gammaproteobacteria had been dominant. Presence of class Alphaproteobacteria did not depend on chloramine residuals or nitrification activity.

Therefore, both autotrophs (nitrifiers) and heterotrophs can be identified as dominant in chloraminated systems. Furthermore, nitrifiers produce extra polymeric substances (EPS) or/and soluble microbial products (SMP) (Kindaichi, Ito & Okabe 2004; Ohashi et al. 1995; Rittmann, Bruce E, Regan & Stahl 1994) which are mainly consisting of polysaccharides, proteins, nucleic acids and lipids (Flemming & Wingender 2010; Sheng, Yu & Li 2010) in certain scenarios. Other than nitrifiers, heterotrophic bacteria such as *Mycobacteria* (Chapman 2003) and *Methylobacterium* (Tsagkari & Sloan 2018) produce EPS in drinking water systems. As CDP is produced/released by micro-organisms living in chloraminated drinking water, they can be accountable for EPS.

The NOM, which is measured as DOC has seasonal variation even on the same location (Matilainen, Vepsäläinen & Sillanpää 2010) and the CDP impact varies with DOC concentration (Herath, Torres & Sathasivan 2018). Therefore; in controlling CDP, it is important to investigate microbial community variations in reactor sets containing different DOC levels which represent the real water distribution system. In the current study, bacterial community presented in reactor sets operated with chloraminated water containing different DOC levels (0-1, 2-3, 4-5 and 7-8 mg-C.L^{-1}) were characterised. The community composition was reported with respect to various water quality parameters (DOC, total chlorine, ammonia, nitrite).

5.2 Materials and methods

5.2.1 Sample collection for bacterial community analysis

Samples were collected from the same reactor sets described in the chapter 4 (section 4.2) at the same time when the samples were collected for chloramine decay tests conducted in the chapter 4. Feed water collection and preparation for the sample collected reactor sets were identical as described in the chapter 4. There were 4 reactor sets fed with different DOC levels of 0-1, 2-3, 4-5 and 7-8 mg-C.L^{-1}.

5.2.2 Chloramine decay rates and their relation to AOB activities

Total chloramine decay (decay due to interactions with compounds in bulk water, sediment and biofilms) coefficient in each reactor was calculated using Eq. 5.1 as defined by Sathasivan, Fisher and Kastl (2010).

$$Cl_{out} = \frac{Cl_{in}}{(1+K_{Rt}\theta)}$$ (Eq. 5.1)

where, Cl_{out} and Cl_{in} are outlet and inlet chloramine (as total chlorine) residuals respectively, k_{Rt} is the total chloramine decay coefficient and Θ is the water retention time in the reactor.

5.2.3 Determination of AOB activities

AOB activity was calculated in each reactor using Eq. 5.2. NOx (nitrite and nitrate) production in each reactor was used to determine the AOB activity, as only AOB are known to increase NOx residuals through ammonia oxidation. Non biological mechanisms of ammonia oxidation are not predominant (Valentine, R et al. 1987).

$$NO_{X\,out} = \frac{NO_{X\,in}}{(1+K_N\theta)}$$ (Eq. 5.2)

where, NO_{Xout} and NO_{Xin} are outlet and inlet NOx-N concentration, respectively. k_N is the AOB activity and Θ is the water retention time in the reactor.

5.2.4 Sample preparation and DNA extraction

After the 6 months of the operational period, 2 L of bulk water from each reactor with different DOC levels were collected in a autoclaved glass bottle. The sampling tap was kept open for 5 s to remove the water inside the pipe lines prior to the bulk water collection. Total chlorine (TCl), total ammoniacal nitrogen (TAN), DOC, pH, nitrite and nitrate were measured immediately after collecting the samples. Water samples (2 L) were filtered through a 0.22 μm filter paper (Nitrocellulose membrane, GSWP03700, Merck Millipore Ltd, Tullagreen, Carrigtwohill Co. Cork, IRL) to concentrate the biomass. The collected biomass on the filter paper was carefully scrapped in to an autoclaved 2 mL eppendorf tube.

DNA was then extracted from the concentrated biomass using the QIAamp® DNA mini kit (Cat. No. 51304, QIAGEN GmbH, D-40724 Hilden) following the manufacturer's instructions. Before the samples were being analysed further, DNA purity was measured using Nano Drop 2000c spectrophotometer. A ratio of absorbance A260/230 greater than 1.8 is typically considered suitable for analysis. This analysis was performed with 1 µL of DNA sample. The extracted samples were kept in a freezer (< -20 ºC) until outsourced for sequencing.

5.2.5 Bacterial community characterization using Miseq

The DNA extractions were then stored at -20 °C prior to shipment to MR DNA (Molecular research LP, Texas, USA) for Illumina MiSeq sequencing. Shipment was done at room temperature on stabilizing the DNA using DNA stable Plus (Biometrica, Diagnostic Technology). Sequencing was carried out using MiSeq Illumina sequencing platform. In brief, HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used for a single-step 28 cycle PCR amplification using 16S universal bacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT). The thermocycler conditions used included an initial denaturing followed by 28 cycles of denaturation at 94 °C for 30 s; annealing at 53 °C for 40 s; and an elongation at 72 °C for 1 min; and a final elongation step at 72 °C for 5 min. After amplification, PCR products were checked in agarose gel (2%) to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare Illumina DNA library. Sequencing was performed at MR DNA (www.mrdnalab.com) on a MiSeq following the manufacturer's guidelines.

5.2.6 Post sequencing process

Post sequencing process of data was carried out using Quantitative Insights into Microbial Ecology (QIIME) (Caporaso, Kuczynski, et al. 2010) software package (http://www.qiime.org). Maximum sequence length was set at 600 and fast qual and mapping files were used as input for split_libraries.py script with default arguments to extract sequences relevant to this study. Then all sequences were groups into operational taxonomic units (OTUs) using pick_otus.py script in QIIME using USEARCH method (Edgar 2010). The OTUs were

defined at 97% sequence similarity and minimum cluster size was 1. Then using the PyNAST default method a representative sequence from each OUT was selected and aligned against Greengenes imputed core reference alignment using align_seqs.py script (Caporaso et al. 2010). The gaps of the aligned sequence were removed using script filter_alignment.py. With default settings- Fast Tree phylogenetic tree and Ribosomal Database Project classifier and Greengenes OTUs data set- taxonomy assignment were constructed using the script make_phylogeny.py and script assign_taxonomy.py, respectively.

To analyse different aspects of microbial community following were derived using alpha_rarefaction. py script, assess the bacterial coverage- rarefaction curve, estimate species richness- Chao 1, phylogenetic coverage of OUT- phylogenetic diversity (PD), bacterial diversity- Shannon index and statistically estimate similarities between clone libraries- Jaccard index.

Canonical correspondence analysis (CCA) was used to discover how environmental factors related to the development of bacterial communities in the reactors. Matching correspondences between three reactors with cluster analysis using PAST software was performed using Bray-Curtis similarity index (based on shared OTUs having 97% sequence similarity) and revealed the differences and similarities of bacterial communities among reactors.

5.3　Results and discussion

5.3.1　Characteristics of collected water samples

The corresponding reactors of all lab scale reactor sets behaved in almost similar ways. Therefore, the chemical composition of reactors (R-1 to R-5) operated by feeding water with 4-5 mg-C.L^{-1} DOC has been explained. The observed parameters of all water samples tested are recorded in Table 5.1. The total chlorine residuals were differing significantly from R-1 to R-5 with the associated nitrogenous compounds. Progressive decay of total chlorine residuals maintained at 1.61 mg.L^{-1} in R-1 was observed along the reactors and over a 4-day retention period, the residuals had dropped down to 0.05 mg.L^{-1} on reaching R-5 (Table 5.1). Loss of TAN and the production of nitrite and nitrate in all reactors were detected with this pronounced drop of chloramine.

It was noted that nitrification had mostly commenced from R-2 as rapid changes of NOx production had started from R-2. NOx production indicated the degree of AOB activity and the changes in concentration along reactors R-1 to R-5 were 0.005, 0.034, 0.194, 0.171 and 0.038 mg-N.L^{-1}, respectively. Nitrifying activities are also highlighted due to the pH decrease (Tuovinen et al. 1980) from 8.00 in R-1 to 7.63 in R-5. As noted previously, the maximum CDP production/presence was associated during severe nitrification, such as in R-5, where the highest nitrification was observed (Krishna, Sathasivan & Sarker 2012). The DOC level did not change greatly between the first (R-1) and last (R-5) reactors of each set (Table 5.1). If the CDP is produced at high concentration, the DOC level would have increased when water travelled from R-1 to R-5 as an indication of CDP production. However, such indication was not expressed along the reactors and; hence, CDP must be present at a low concentration; which may not be measured by assessing DOC.

Table 5.1: Physicochemical parameters measured along reactors (R-1 to R-5) in 4 lab scale reactor sets with different DOC levels

Reactors		DOC (mg-C.L^{-1})	Total Chlorine (mg-Cl$_2$.L^{-1})	TAN (mg.L^{-1})	NOx (mg-N.L^{-1})	Nitrite (mg-N.L^{-1})	Nitrate (mg-N.L^{-1})	pH
7-8 (mg-N.L^{-1}) DOC containing reactor set	R-1	7.30±0.37	1.93±0.03	0.56±0.011	0.365±0.018	0.018±0.000	0.347±0.007	8.05±0.10
	R-2	7.28±0.36	1.43±0.03	0.45±0.009	0.479±0.024	0.113±0.002	0.366±0.008	7.85±0.10
	R-3	7.31±0.37	0.63±0.03	0.16±0.003	0.682±0.034	0.292±0.006	0.390±0.008	7.76±0.10
	R-4	7.23±0.36	0.13±0.03	0.05±0.001	0.818±0.043	0.412±0.008	0.406±0.009	7.64±0.10
	R-5	7.35±0.37	0.04±0.03	0.02±0.000	0.864±0.045	0.454±0.008	0.410±0.009	7.61±0.10
4-5 (mg-N.L^{-1}) DOC containing reactor set	R-1	4.35±0.22	1.61±0.03	0.50±0.010	0.381±0.019	0.005±0.000	0.376±0.008	8.00±0.10
	R-2	4.29±0.21	1.37±0.03	0.44±0.009	0.415±0.021	0.034±0.001	0.381±0.008	7.87±0.10
	R-3	4.23±0.21	0.31±0.03	0.21±0.004	0.609±0.030	0.213±0.004	0.396±0.008	7.75±0.10
	R-4	4.29±.021	0.09±0.03	0.07±0.001	0.780±0.039	0.378±0.008	0.402±0.008	7.69±0.10
	R-5	4.28±0.21	0.05±0.03	0.04±0.001	0.818±0.041	0.405±0.008	0.413±0.008	7.61±0.10
2-3 (mg-N.L^{-1}) DOC containing reactor set	R-1	2.65±0.13	2.01±0.03	0.59±0.012	0.401±0.015	0.002±0.000	0.399±0.006	8.01±0.10
	R-2	2.60±0.13	1.46±0.03	0.48±0.010	0.433±0.017	0.030±0.001	0.403±0.006	7.91±0.10
	R-3	2.63±0.13	0.48±0.03	0.099±0.002	0.658±0.035	0.230±0.004	0.428±0.009	7.81±0.10
	R-4	2.58±0.13	0.08±0.03	0.07±0.001	0.751±0.033	0.273±0.005	0.478±0.008	7.85±0.10
	R-5	2.63±0.13	0.03±0.03	0.03±0.001	0.788±0.034	0.293±0.006	0.495±0.008	7.79±0.10
0-1 (mg-N.L^{-1}) DOC containing reactor set	R-1	0.29±0.01	1.78±0.03	0.49±0.010	0.360±0.018	0.002±0.000	0.358±0.007	8.06±0.10
	R-2	0.56±0.03	1.28±0.03	0.36±0.007	0.372±0.019	0.003±0.000	0.369±0.007	7.85±0.10
	R-3	0.23±0.01	0.35±0.03	0.07±0.001	0.677±0.034	0.198±0.004	0.479±0.010	7.71±0.10
	R-4	0.85±0.04	0.05±0.03	0.04±0.001	0.761±0.038	0.283±0.006	0.478±0.010	7.64±0.10
	R-5	0.74±0.04	0.03±0.03	0.03±0.001	0.789±0.039	0.289±0.006	0.500±0.010	7.62±0.10

5.3.2 Chloramine decay rates and their relation to AOB activities

Fig. 5.1 illustrates chloramine decay rates and AOB activities in reactor sets with different DOC levels. In all reactor sets, chloramine decay rate coefficients behaved in similar manner by increasing up to R-4 and then showing a sudden drop in R-5. AOB activities followed the same pattern up to R-3, and then the activity decreased in R-4 and R-5. Due to the low chloramine levels in R-4 and R-5 (0.02-0.07 mg-Cl_2.L^{-1}), the decay rate coefficient recorded in low levels (Krishna, Sathasivan & Ginige 2013). In this study, this pattern is followed by the R-5, but not by R-4 and instead all R-4 reactors of all sets recorded higher decay rate. This unexplainable decay rate could be due to the CDP activity. However, as in higher DOC levels did not recorded high CDP activity (Chapter 4), the resulted higher decay rate with R-4 in 7-8 mg-C.L^{-1} DOC level could be due to some other unknown activities. As high DOC suppresses the CDP activity, the maximum decay rate observed in the reactor sets operated with 7-8 mg-C.L^{-1} DOC was only 0.16 h^{-1} while the other DOC levels recorded more than 0.2 h^{-1} decay rate. Low free ammonia residuals in R-4 and R-5 could be the reason for reduced AOB activities. If the chloramine residuals and free ammonia residuals were high in reactors, higher AOB activity and decay rate co-efficient could be observed.

With the reduction of DOC levels in reactor sets, the maximum AOB activity increased. The increased in AOB activities with decreasing DOC is more pronounced in R-3 of each set. Therefore, AOB activity would be suppressed by the higher DOC levels as heterotrophic bacterial colonies would dominate the community composition.

(a)   (b)

(c)   (d)
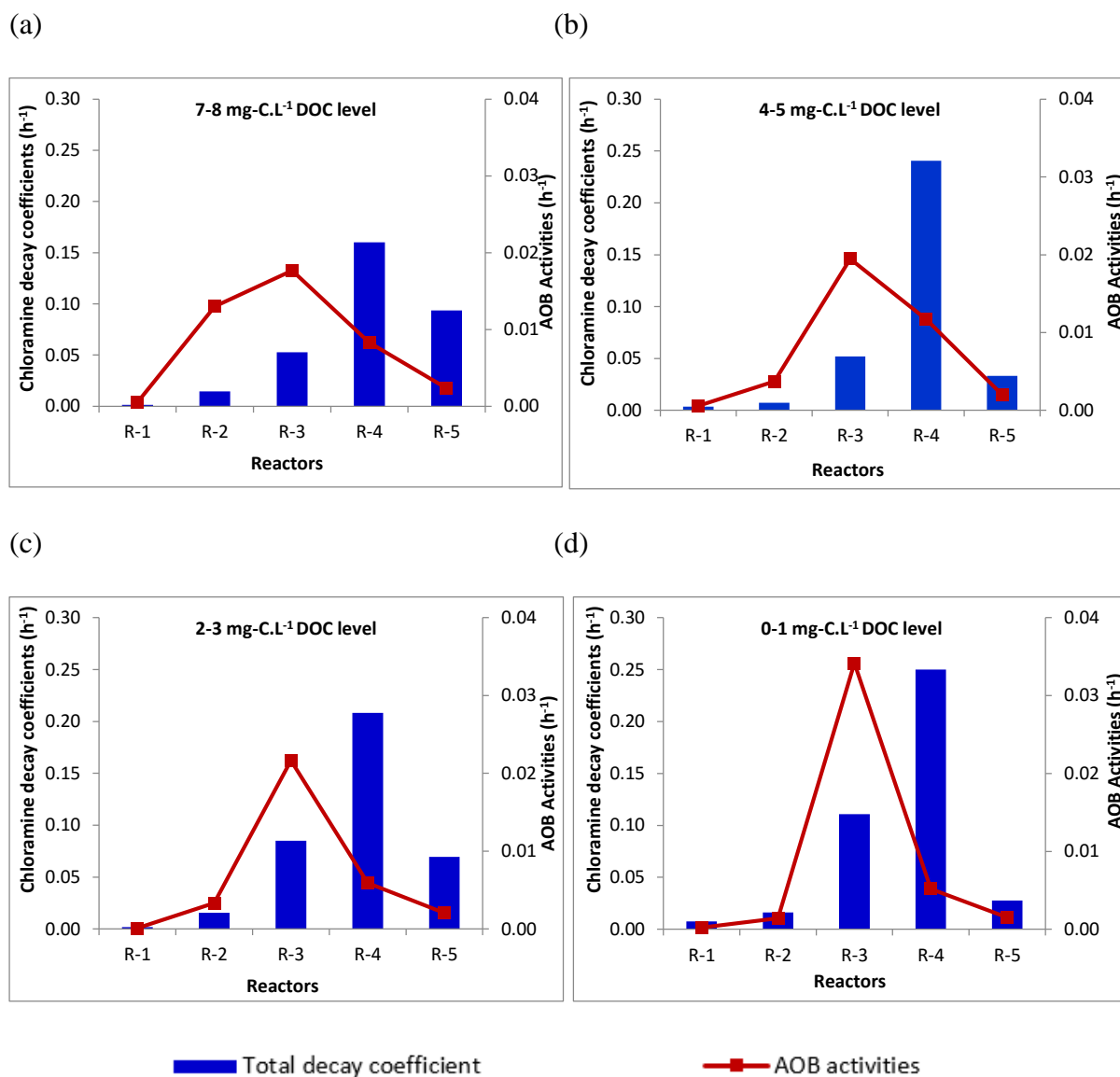
Figure 5.1: Total chlorine decay rate coefficients in each reactor and their relationship with AOB activities along reactor (R-1 to R-5) in variable DOC contained reactor sets

5.3.3 Microbial community structure analysis

Diversity analysis

The rarefaction analysis (Fig. 5.2) yields insights into the sequencing effort and compares the diversity of the observed number of OUTs at 97% sequence similarity in all samples over time. The samples analysed showed a steep slope in rarefaction curves suggesting that further sequencing would be needed to reach a full taxonomic representation of the microbial comminities. A total 15,454,255 sequences were generated from 19 different samples and after

processing (removing short sequences and chimera) 3,037,350 sequences (94% of the total sequences) were used to examine bacterial diversity. Diversity or taxonomic analysis for the R-3 from 2-3 mg-C.L^{-1} DOC level could not be generated due to the low concentration of DNA.
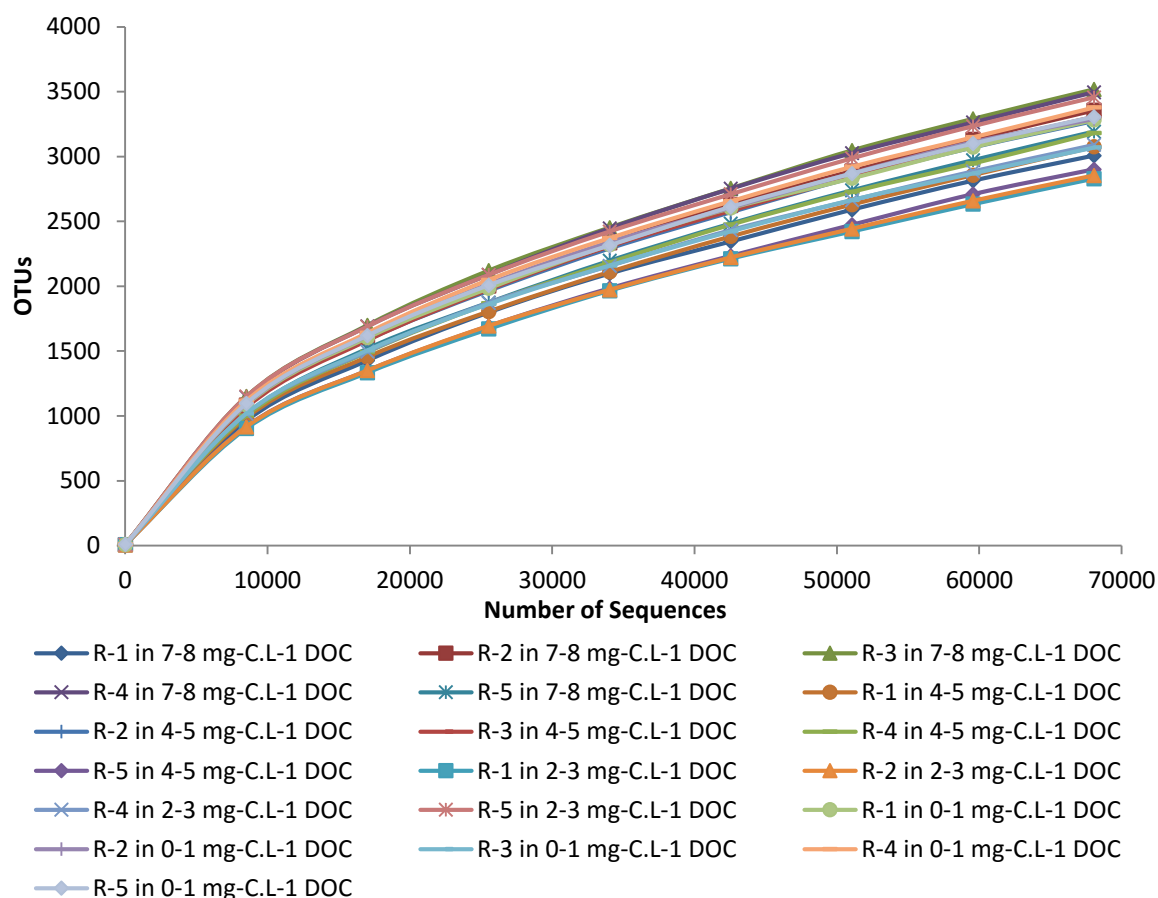
Figure 5.2: Rarefaction analysis of microbial communities at 97% sequence similarity of the reactors with different DOC levels

Table 5.2 shows the richness (i.e. number of different OTUs) and diversity (i.e. number of different OTUs taking in to account their relative abundance) of the bacterial communities in collected samples at 97% sequence similarity. When reactor sets were compared, the highest species richness and pylogenic diversity showed in 7-8 mg-C.L^{-1} DOC containing reactor set while 2-3 mg-C.L^{-1} DOC reactor set indicated the lowest species richness. Species diversity not drastically deviate among the reactor sets. However, in 7-8 and 4-5 mg-C.L^{-1} DOC levels, the highest species diversity were recorded in middle 3 reactors (R-2 and R-3 and R-4) while the first and the last reactors consisted with the lower diversity.

Table 5.2: Bacterial diversity indices of samples collected from different DOC level contained reactor sets

samples		Chao1[*] (average)	Shanon Index[*]	PD[*] (average)
7-8 (mg-C.L^{-1}) DOC containing reactor set	R-1	6028.03	6.49	182.12
	R-2	6503.24	7.20	200.55
	R-3	6508.44	7.60	210.58
	R-4	6251.86	7.74	206.02
	R-5	6153.42	6.83	187.26
4-5 (mg-C.L^{-1}) DOC containing reactor set	R-1	6053.26	6.76	201.39
	R-2	6016.63	7.53	190.09
	R-3	6030.00	7.61	195.25
	R-4	6084.18	7.29	196.93
	R-5	5882.71	6.32	184.59
2-3 (mg-C.L^{-1}) DOC containing reactor set	R-1	5395.96	6.41	167.40
	R-2	5527.16	6.08	180.46
	R-4	5777.51	7.20	187.02
	R-5	6390.24	7.73	203.22
0-1 (mg-C.L^{-1}) DOC containing reactor set	R-1	5775.97	7.43	191.94
	R-2	6097.83	7.35	195.98
	R-3	5509.35	6.82	176.62
	R-4	6201.30	7.11	198.32
	R-5	5887.71	7.86	196.40

[*] Sequence per sample normalized to 59559

Similarity analysis

CCA at the genus level (based on the Jaccard similarity matrix at 80% sequences similarity) using physiochemical parameters is presented in Fig. 5.3a. The analysed physiochemical variables that included in the CCA analysis were DOC, NOx, TAN and total chlorine. However, there were no clear correlation can be witnessed between DOC levels and NOx, TAN or total chlorine in relation to bacterial community distribution.

(a)
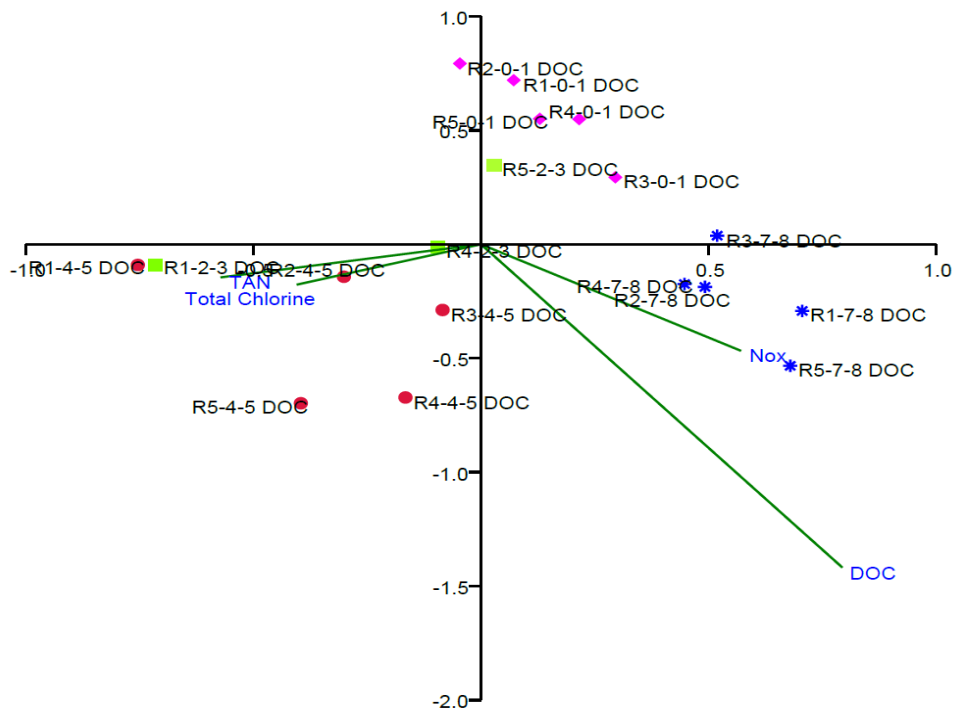
(b)
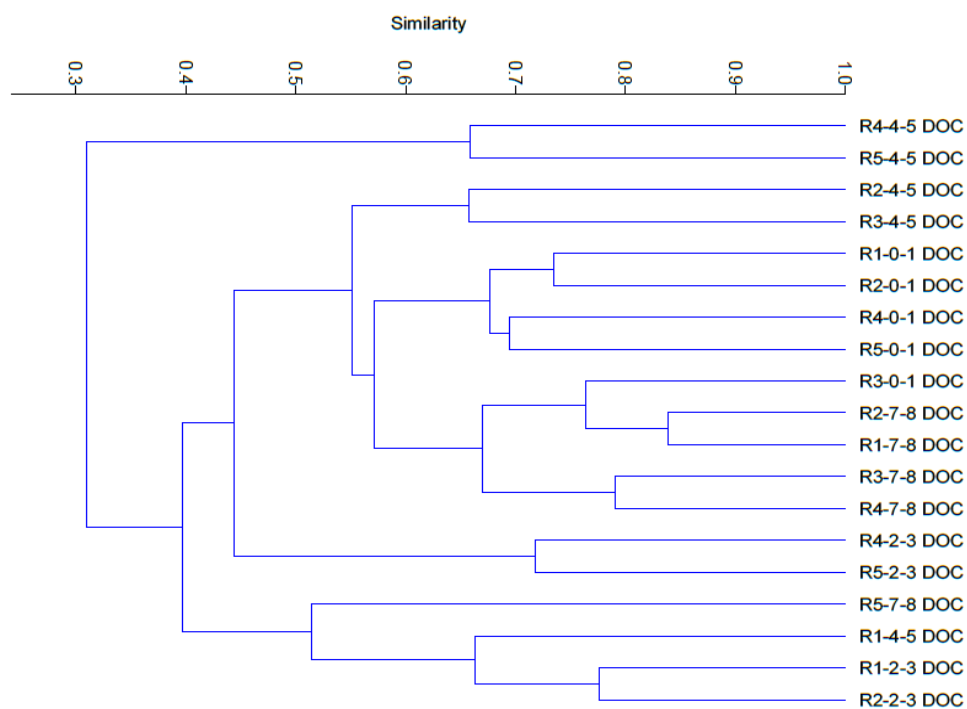
Figure 5.3: A similarity analysis (at 80% sequence similarity) of reactor samples maintained with different DOC levels at genus level based on Jaccard similarity index. (a) Canonical correspondence analysis (CCA); (b) Cluster analysis.

According to the cluster analysis (Fig. 5.3b) at the genus level, there were clear differences that were detected based on DOC levels. In most cases, the reactors included in the same DOC level containing lab scale reactor set had clustered together with high similarity. In the samples collected from the reactors within the 4-5 mg-C.L^{-1} DOC level containing reactor set, R-2-R-5 showed 66% similarity. However, R-1 was clustered with R-1 and R-2 (with70% similarity) of the 2-3 mg-C.L^{-1} DOC level containing reactor samples. In the samples collected from the 0-1 mg-C.L^{-1} DOC contained reactor set, the reactor samples (except R-3) were clustered together with 70-75% similarity. Among all samples, R-1 to R-4 of 7-8 mg-C.L^{-1} DOC level reactor set clustered together with the similarly 70%. Even though all 5 reactor samples from the same DOC contained reactor set were not clustered together, the majority had stayed together due to the presence of similar DOC levels. The feed water for 2-3 mg-C.L^{-1} DOC level had been prepared by diluting the 4-5 mg-C.L^{-1} DOC water with ultra-pure water in 1:1 ratio. This could be the reason for clustering in R-1 samples together from both reactor sets at 65% similarity.

Taxonomic analysis

The dominant phyla (with relative abundance >2% in any given sample) is presented in Fig. 5.4. The most abundant bacterial phyla among all reactors with different DOC levels was the Proteobacteria. The first 3 reactors having DOC levels of 7-8 recorded more than 88% of Proteobacteria . However, R-5 had only 50% relative abundance. All the reactors with 0-1 mg-C.L^{-1} DOC had experienced high level of Proteobacteria (74-90%). Lowest levels of proteobacteria were recorded in R-4 and R-5 (48% and 36%) in the 4-5 mg-C.L^{-1} DOC level. In 2-3 DOC level, Proteobacteria and Actinobacteria occupied almost all bacterial phyla in the R-1 (55% and 43%, respectively) and R-2 (45% and 49%, respectively). Cyanobacteria had been observed in all reactors in range of DOC levels, although in low persentages. Maximum Cyanobacterial relative abundance were recorded in R1(8%) and R-2 (7%) in 0-1 mg-C.L^{-1} DOC level and R1 in 4-5 mg-C.L^{-1} DOC level , while in other reactors they wer < 3%. Plancomycetes were high within the 7-8 mg-C.L^{-1} DOC level having a maximum of 24% in R-5. This could be caused by the presence of high DOC level, which could promote the growth of plancomycetes. In all other DOC levels, the plancomycetes levels were at less than 3 %. However, It is reported that the members of this bacterial class are phylogenetically indecipherable (Chistoserdova et al. 2004). In plyla Bacteroidetes, the only considerable abundance was recorded in R-4 (24%) and R-5 (40%) within the 4-5 mg-C.L^{-1} DOC level,

where they were the second most abundant bacterial phyla. To understand indepth details about how these phyla are connected with CDP production and fast decsay of chloramine, bacterial communities were further analysed at class level and genera level as described in below.
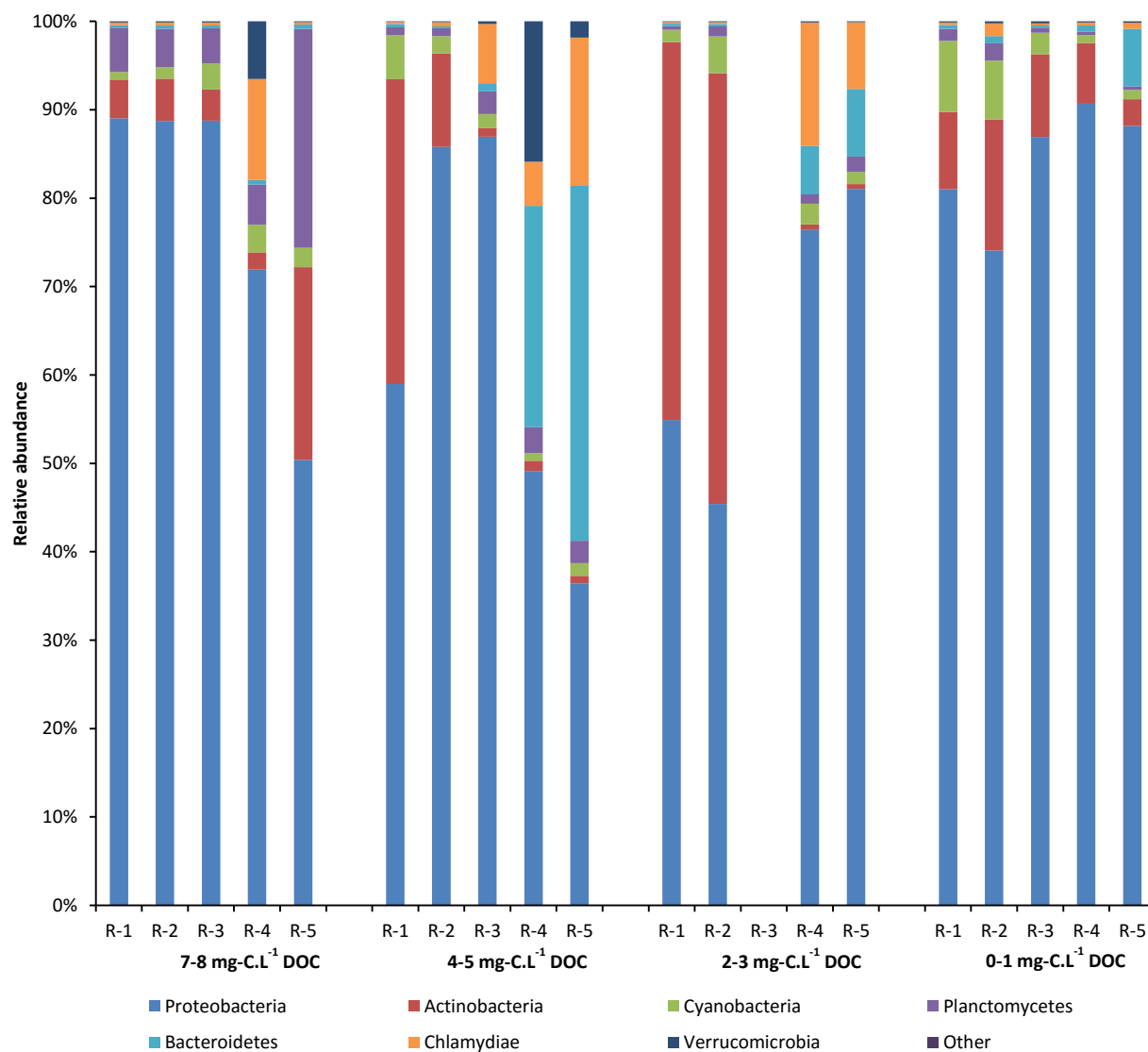
Figure 5.4: Relative abundance of bacterial communities at Phyla level in 19 reactors operated with different DOC level containing chloraminated water. Bacterial community of R-3 (2-3 mg-C.L^{-1}DOC level) was not reported.

The decrease of chloramine and TAN residuals and the increase of NOx along the reactors resulted with remarkable changes in the bacterial communities in different DOC levels. The most abundant bacterial class among all reactors in all DOC levels was Alphaproteobacteria, which belongs to the heterotrophic bacterial community. The highest abundance was recorded

in 4-5 mg-C.L^{-1} DOC level (75% in R-2 and 82% in R-3) and 7-8 mg-C.L^{-1} DOC level (74% in R-1 and 73% in R-2) (Fig. 5.5).
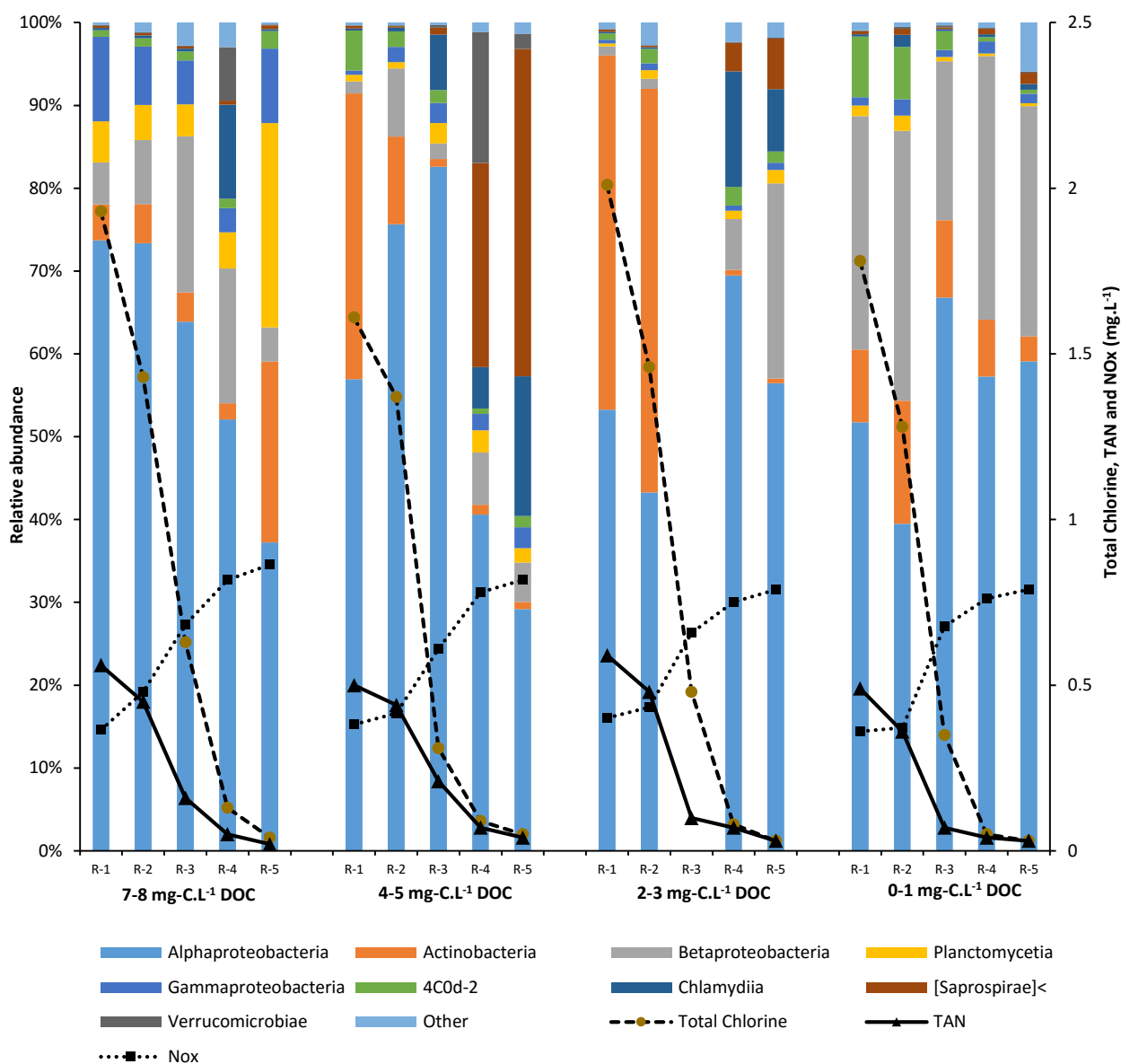
Figure 5.5: Relative abundance of bacterial communities at class level in 19 reactors operated with different DOC level containing chloraminated water. Bacterial community was not reported to R-3 in 2-3 mg-C.L^{-1}DOC level.

Class Actinobacteria was observed as prominent in R-5 and recorded minor levels in all other reactors with 7-8 DOC mg-C.L^{-1}. In all other reactor sets, high relative abundance of Actinobacteria were observed in the first two reactors where chloramine residuals were high. It is a well-known bacterial class which resist high chlorine residuals (Li, X et al. 2014) and

highly dominant in both chlorinated and chloraminated distribution systems (Holinger et al. 2014).

AOB belongs to class Betaproteobacteria and AOB abundances were higher in the nitrifying conditions. However, in 7-8 mg-C.L^{-1} DOC, Betaproteobacteria was high even with low NOx levels (R-1 and R-2, 16% and 19 %, respectively) as all Betaproteobacteria are not nitrifiers. The highest abundance of Betaproteobacteria were observed in the reactors of 0-1 mg-C.L^{-1} DOC. Moreover, according to the results obtained by Herath et al (2018), the highest impact CDP was observed in the reactors maintained with 0-1 mg-C.L^{-1} DOC. This gives a clue that Betaproteobacteria may responsible for this CDP production. However, further investigations need to be carried out.
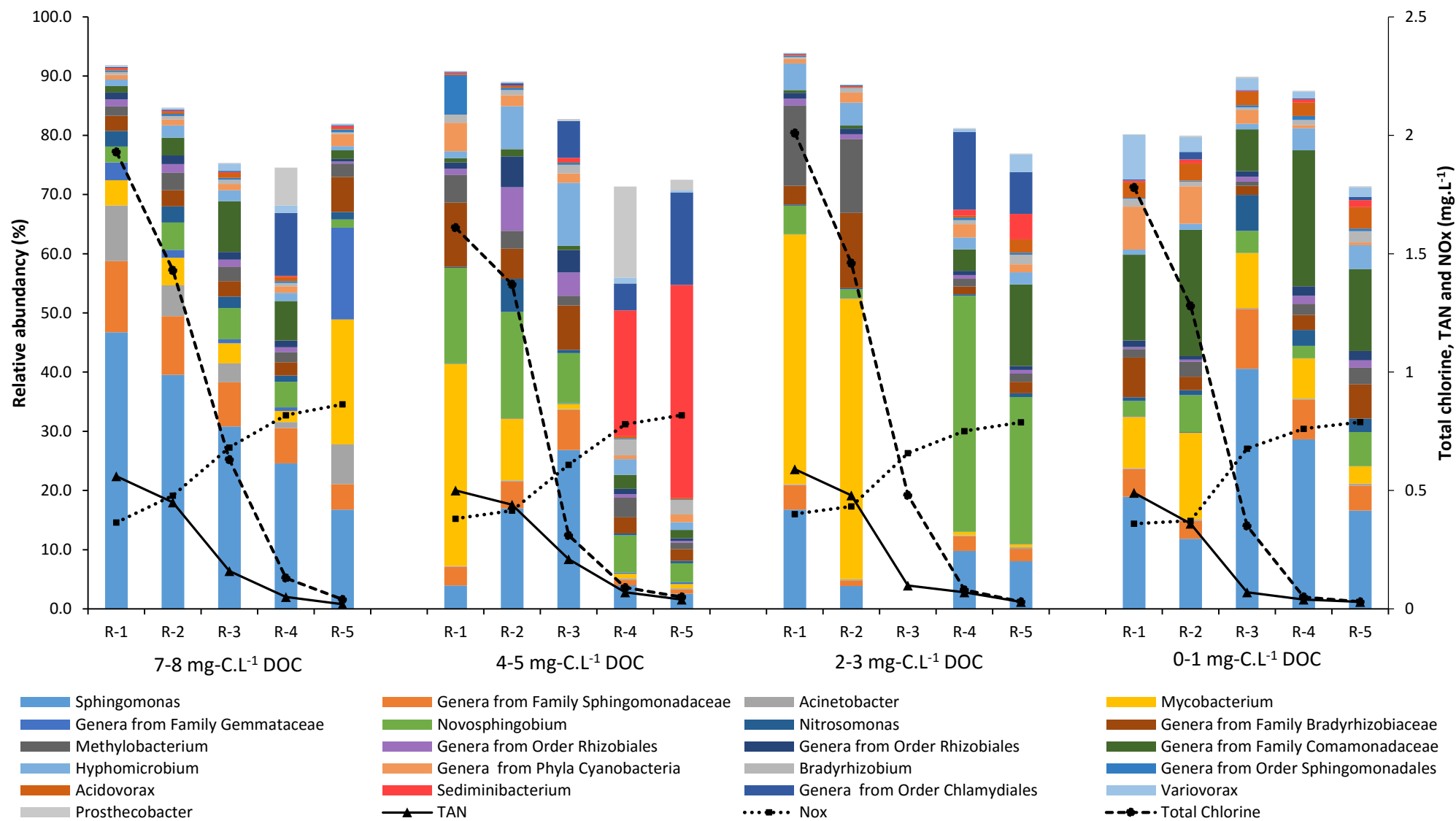
Figure 5.6: Relative abundance of bacterial communities at genus level in 19 reactors operated with different DOC level containing chloraminated water. Bacterial community of R-3 (2-3 mg-C.L^{-1}DOC) level was not reported.

Several bacterial genera are known to contain in reactors with different DOC levels contained lab scale reactor sets. Fig. 5.6 shows an array of bacterial genera belonging to Alphaproteobacteria (*Sphingomonas,* genera from order Rhisobiales, *Hyphomycrobium, Phenylobacterium, Afipia*), Betaproteobacteria (*Nitrosomonas, Acodovorax, Varouvarax, Methylibium,* genera from family comamonadaceae), Gammaproteobacteria (*Acinetobactor* and genera from family Legionellaceae), Actinobacteria (*Mycobacterium*), Plancomycetia (genera from family Gammataceae), Saprospirae (*Sediminibacterium*), Chlamydiia (genera from order Chlamydiales) and Virrucomicrobiae (*Prosthecobactor*). Relative abundance differs according to the DOC level, chloramine residual and the microbial metabolites such as NOx.

In 7-8 and 0-1 mg-C.L^{-1} DOC levels, *Sphingomonas* was the prominent bacterial genera among all reactors except R-2 in 0-1 mg-C.L^{-1} DOC. In R-1 with 2-3 mg-C.L^{-1} DOC level high *Sphingomonas* (17%) was detected. *Sphingomonas* is gram-negetive, chemoheterotrophic, strickly aerobic bacterium, which is commonly isolated in drinking water systems (Koskinen et al. 2000). As it survived even with high chloramine contained water (Sun et al. 2013), it seems that abundancy does not depend on the disinfectant level. It is also not influenced by the DOC level of the reactor water as it was presented in the same abundance in both high and low DOC contained water. However, due to the reduction of chloramine and high DOC level favouring the growth of other bacterial spp, their abundance had reduced from R-1 to R-5 in 7-8 mg-C.L^{-1} DOC.

In 0-1 mg-C.L^{-1} DOC level, the low abundance of *Sphingomonas* in R-1 and R-2 could be due to low DOC level. However, during the reactor operational period, number of dead cells along reactors could increased and therefore; high level of assimilable organic carbon (AOC) could be produced . Therefore, from R-3 to R-5, *Sphingomonas* demonstrated increacing abundancy. Additionally, in 4-5 and 0-1 mg-C.L^{-1} DOC levels, the highest relative abundance were recorded in the R-3 where the nitrification had commenced. These results are correspondant with the results obtained by Krishna, Sathasivan and Ginige (2013). Similarly, it has been reported the dominence of this genus in chloraminated systems ((Noguera et al. 2009; Regan, John M et al. 2003; Regan, John M, Harrington & Noguera 2002; Williams et al. 2004). Another unclassified bacterial type belonging to family sphinogomanadaceae was destributed in the reactors in the similar pattern.However their precence is almost 4 times lower than the *Sphingomonas*.

Novosphingobium was identified as another heterotrophic bacterial genera belonging to the same taxonomic family (Sphingomonadaceae) in seed and chloraminated reactor samples. It is a genus of Gram-negative bacteria and some genera (e.g. *Novosphingobium taihuense, Novosphingobium aromativorans*) can degrade aromatic compounds such as phenol, aniline, nitrobenzene and phenanthrene (Liu, Z-P et al. 2005; Sohn et al. 2004). It has been detected in chlorinated drinking water systems as one of the abundunt genera (Gomez-Alvarez et al. 2016). In this study, it was the most abundant bacterial genus in R-4 (40%) and R-5 (25%) with 2-3 mg-C.L^{-1} DOC. In 7-8 and 0-1 mg-C.L^{-1} DOC levels, *Novosphingobium* was detected in lower amounts (< 1%) and their abundance was almost equally distributed among all reactors. However, their ability to degrade aromatic compounds can be related to chloramine decay as they are one of the common bacterial type in drinking water destribution systems.

(a)   (b)

(c)   (d)
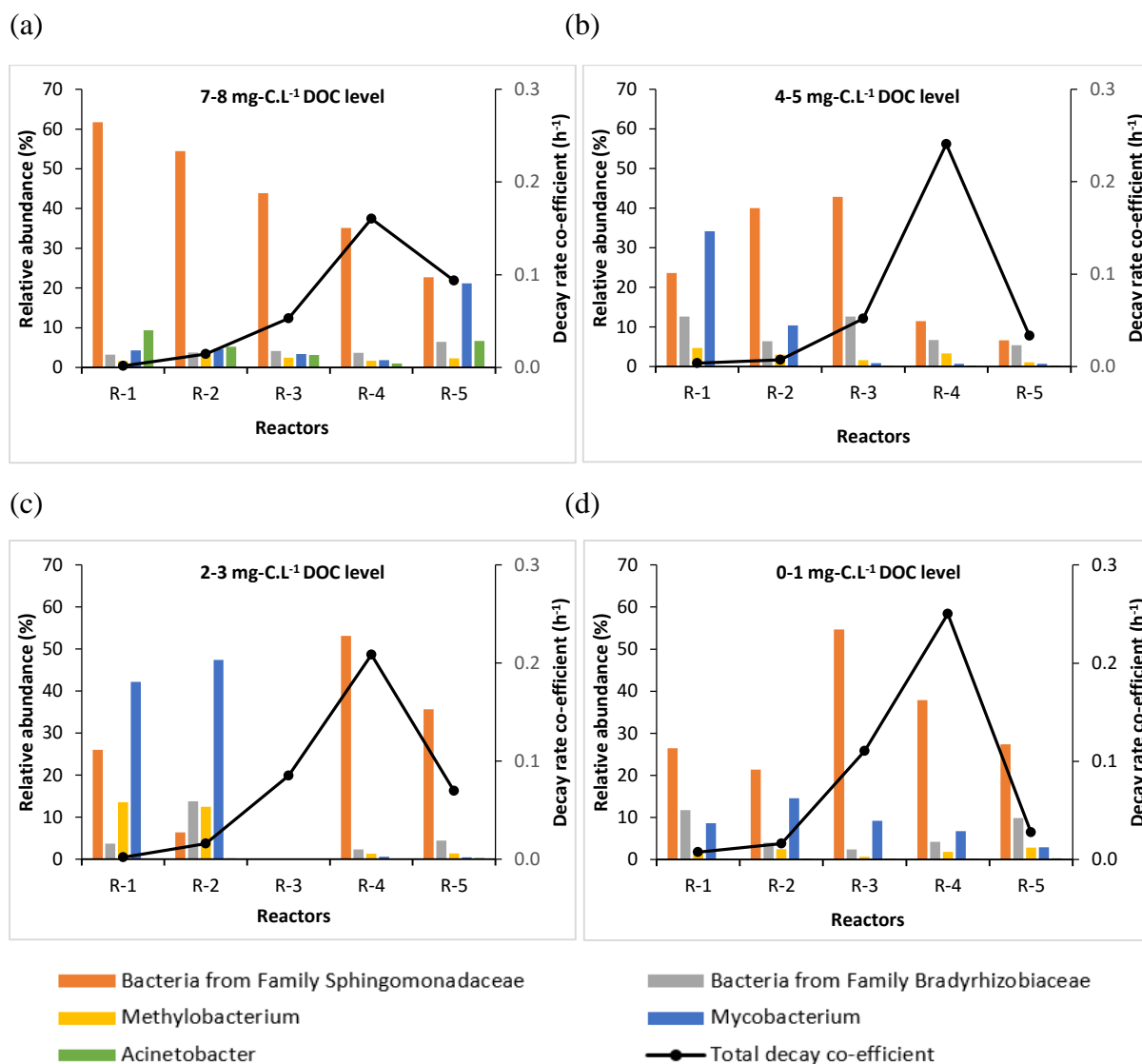
Figure 5.7: Relationship between total decay rate coefficient and relative abundance of identified bacteria

Many Sphingomonadaceae bacteria can produce abundant EPS (Liu, R et al. 2012), which could relate to the CDP. However, relative abundance of the bacteria belongs to family Sphingomonadaceae does not idicate strong relationship with the total decay co-efficient (Fig. 5.7) in reactors of each reactor sets. In overall, when the total decay co-efficient decreased in the last two reactors of each reactor set, relative abundance of bacteria belongs to family Sphingomonadaceae also reduced. As there was no cell number details in reactors/reactor sets, their relatoinship with DOC level cannot be confirmly demonstrated.

Other than the bacteria belongs to family sphingomonadaceae, *Mycobacterium* was prominent in R-1 and R-2 with 2-3 mg-C.L^{-1} DOC level (42% and 47%, respectively) and with 4-5 mg-C.L^{-1} DOC level (34% and 10%, respectively) when there was high chloramine level present.

Reduction of *Mycobacterium* from R-3 (<0.1%) onwards could be due to the invasion of fast growing bacteria such as Bradyrhizobium, family sphingomonadaceae according to the current study . Similarly, reactors maintained with 0-1 mg-C.L^{-1} DOC demonstrated the same pattern along with *Mycobacterium* abundance. However, when the chloramine level dropped below 0.5 mg-Cl$_2$.L^{-1}, there were no drastic reduction of *Mycobacterium* community from R-3 onwards as noted in 2-3 and 4-5 mg-C.L^{-1} DOC levels. *Mycobacterium* is a widespread organism, typically living in water (including tap water treated with chlorine) and soil (Torvinen et al. 2004). They are more tolerant of chlorine than many other microbes (Carson et al. 1978; Covert et al. 1999; Le Dantec et al. 2002; Pelletier & Stottmeier 1988; Taylor et al. 2000), and increased abundance of *Microbacteria* in chloraminated distribution systems has been reported by Noguera et al. (2009). They have also been isolated from drinking water with free chlorine content up to 2.5 mg.L^{-1} (Covert et al. 1999). The observed result is consistant with the results witnessed by (Krishna, Sathasivan & Ginige 2013) as they also detected high *Microbacterium* relative abundance with high chloramine residuals. Recent studies carried out by Wang, Hong et al. (2012) confirmed that *Mycobacterium* could be existing within drinking water systems even in the presence of high chloramine residuals (> 5 mg.L^{-1}). Essentially, they were detected as biofilm producing bacteria (Emtiazi et al. 2004), which can secrets EPS.

The second most abundant genera recorded in 0-1 mg-C.L^{-1} DOC level was a genus from the family of Comamonadaceae. Drinking water, lake water, activated sludge, soil often contain members of the Comamonadaceae group. Comamonadaceae use a large variety of substrates, hence it is difficult to conclude which substrate was causing the increase in the Comamonadaceae population within the drinking water network (Burtscher et al. 2009; Pernthaler et al. 1998). However, Pinto et al. (2014) reported some species such as *Hydrogenophaga, Acidovorax* from the family Comamonadaceae as one of the abundant betaproteobacterial groups in drinking water systems. *Varivorax* which was detected at higher levels in 0-1 mg-C.L^{-1} DOC also belongs to the family Comamonadaceae. In higher DOC levels such as 7-8 and 4-5 mg-C.L^{-1}, *Varivorax* abundance was < 1%. *Varivorax* is one of the common microbial community members, which is also identified abundanlty in young biofilms in chlorine treated drinking water systems (Douterelo, Fish and Boxall (2018). However, their abundancy related to the DOC level is not known. *Acidovorax* is another major heterotrophic bacterial group identified in drinking water biofilms (Critchley et al. 2003) which belongs to the family Comamonadaceae. EPS is identified as the major constituent of biofilms and as described earlier, CDP can also be accountable for one of the compound in EPS. Therefore,

presence of family Comamonadaceae inrelation to the preduction of CDP has to be further studied.

In addition, uncharacterised bacteria from family Bradyrhizobiaceae had presented in all DOC levels. Other than this bacterial genus 2 uncharacterised bacterial genera belonging to order Rhizobiales were identified with high abundance in R-2 and R-3 maintained with 4-5 mg-C.L^{-1} DOC level but recorded less than 1% in all other reactors including all other DOC levels. Most importantly, *Bradyrhizobium japonicum* was identified for the heat shock protein production (Kishinevsky, Sen & Weaver 1992; Narberhaus et al. 1996). Furthermore, *Bradyrhizobium* strains and Rhizobiales are detected for degrading organophosphorus pesticides by producing extracellular proteins (Abd-Alla 1994). Therefore, in our study they could produce CDP to degrade chloramine as a shock response mechanism.

In many drinking water distribution systems, *Methylobacterium* was identified as one of the most abundant bacterial species (Simoes, Simoes & Vieira 2010). *Methylobacterium sp*. has demonstrated the most resistant biofilms for chlorine and chloramine disinfection as per biofilm removal results they have observed. They are also known to resist free chlorine (Furuhata, K & Koike 1993). Halo acetic acids-HAAs (one of the DBP) have been known to be degraded by drinking water bacteria including *Methylobacterium* (Zhang, Yan & Edwards 2009). Furthermore, chloroform with methane used as available substrate has been capable of being degraded by *Methylobacterium* CRL-26 strain (Cappelletti et al. 2012; Patel et al. 1982). The formation of aggregates in drinking water has been suggested by production of EPS by *Methylobacterium* (Sheng, Yu & Li 2010). *Methylobacterium* species have been previously stated to have an enhanced ability to form aggregates when they are present with other drinking water bacteria (Ramalingam et al. 2013; Simões, LC et al. 2007). Furthermore, *Methylobacterium* strains are known to be multidrug resistant (Furuhata, Katsunori et al. 2006). In this study, *Methylobacterium* could not be observed abundantly (<3%) in 0-1 mg-C.L^{-1} and 7-8 mg-C.L^{-1} DOC levels. However, in 2-3 mg-C.L^{-1} DOC level, R-1 and R-2 were detected with the highest abundancy (14% and 13%, respectively) while R-1 in 4-5 mg-C.L^{-1} DOC fed reactors were detected with 5%.

The observed results could not be interpreted to clearly demonstrate the relationship between identified bacterial strains and the DOC level; as only the relative abundance are being reported. However, the cell numbers along reactors (R-1 to R-5) are drastically increased in the lab scale reactor set experimented by Krishna, Sathasivan and Ginige (2013). Therefore, the

actual numbers could be higher than the recorded relative abundance. As high DOC level promotes heterotrophic bacterial growth (Fonte et al. 2013; Velten et al. 2011), reactors with high DOC levels should facilitate a higher number of cells from each bacterial type compared to cell numbers at lower chloramine level. In a situation of high DOC and low chloramine, the bacterial count will be further elevated. Therefore, the identified heterotrophic bacterial strains with high abundance do not show a correlation with total decay coefficient in individual reactor sets (Fig. 5.7). However, the results may be recorded correctly for the reactor set operated with low DOC level (0-1 mg-C.L^{-1}).

(a)   (b)

(c)   (d)
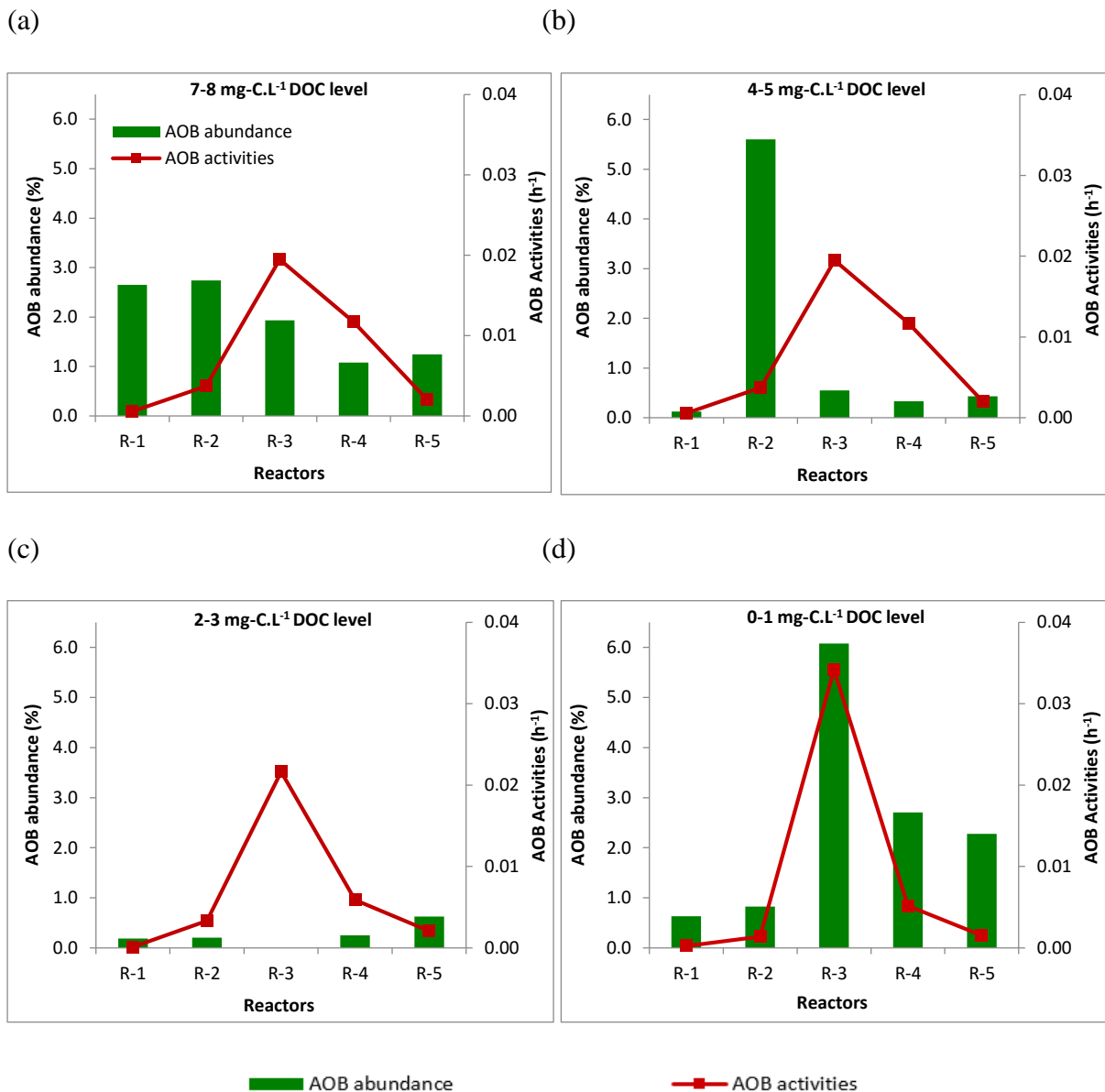
Figure 5.8: AOB activity in relation to their relative abundance

The impact of CDP is mainly observed in the severely nitrified water samples. Therefore, identifying *Nitrosomonas* as one of the AOB in tested samples is highly important in this study. It was predominantly detected in chloraminated drinking water destribution systems (Krishna, Sathasivan & Ginige 2013; Lipponen et al. 2004; Regan, John M et al. 2003; Regan, John M, Harrington & Noguera 2002; Williams et al. 2004; Wolfe et al. 1990). In 4-5 mg-C.L^{-1} DOC level, the highest relative abundance were demonstrated in R-2 (6%) where in the other reactors their abundance were < 0.6%. There was 0.2-0.3% of *Nitrosomonas* spotted in 2-3 mg-C.L^{-1} DOC level. Nevertheless, all reactors in 7-8 mg-C.L^{-1} DOC level were detected with 2-3% *Nitrosomonas* abundancy. According to the results observed, *Nitrosomonas* bacterial community destributed equally with the presence of high DOC level (7-8 mg-C.L^{-1}) without considering the chloramine concentration. However, CDP impact cannot be observed when the DOC level was as high as 7-8 mg-C.L^{-1} (Herath, Torres & Sathasivan 2018). Therefore, as per the obsereved, the production/release of CDP may not be promoted with 7-8 mg-C.L^{-1} DOC level.

Additionally, AOB activity and the AOB abundancy do not indicate corelation except in 0-1 mg-C.L^{-1} DOC level (Fig. 5.8). Similar to heterotrophs, the AOB cell number should increase from R-2 to R-5 as chloramine is dropped significantly and this effect could not be observed on the relative abundance in each reactor . The positive corelation with abuncance and activity in the 0-1 mg-C.L^{-1} DOC level could be related to low DOC level.

In summary; apart from few bacterial types, the majority of bacterial types are similar in reactor sets with different DOC levels. Therefore, CDP producing microbial strains cannot be distinguished by detecting the microbes in variable of DOC levels. However, as it has been observed that the impact of CDP in chloraminated water is supressed due to presence of high DOC level, the necessity has arisen for an experiment utilising low DOC contained chloraminated water. Additionally, production of EPS is boosted when the microbial community is exposed to stress conditions (Liu, Y-Q, Liu & Tay 2004) such as starvation, pH variations and temperature variations. Some of the bacterial strains (e.g. nitrifying bacteria, *Methylobacterium*, *Bradirhizobium*, family Sphingomonadaceae) recognized in this study are also known to produce EPS. Therefore, an experiment using shock application would help recognise the microbes liable for CDP production.

5.4 Conclusion

In parallel to the study carried out to find the effect of feed water DOC variation on CDP production, the bacterial community variations were also analysed on the same reactor sets operated with chloraminated water containing different DOC levels (0-8 mg-C.L^{-1} DOC). The findings of this study are summarised as below:

- DOC variation of feed water did not significantly affect the microbial community present in chloraminated water and the similarities observed between reactors within each reactor set are very high.

- The AOB activity does not correlate with decay rate co-efficient in individual reactor sets. However, AOB activity is highest in the reactor set with lowest DOC level.

- Mixed culture could be responsible for the chloramine decay in each reactor set as there was no single bacterial community could be directly correlated with chloramine decay.

- Some of the bacterial strains such as *Micobacterium*, *Methylobacterium*, *Nitrosomonas*, *Bradyrhizobium sp*. and family Sphingomonadaceae identified are recognised as EPS producing bacteria.

Chapter 6

Microbes resist chloramine by producing chloramine decaying protein

6.1 Introduction

In the prevention of waterborne diseases important roles are played by disinfectants, the most popular being chlorine. Due to the rapid decay of chlorine in drinking water, in addition to difficulties controlling the regulated disinfection by-products (Hoigné & Bader 1988) and further maintenance of adequate disinfectant residuals in distribution systems with long residence times, chlorine is substituted with chloramine as a secondary disinfectant in many countries.

To introduce chloramine into the treated water, ammonia and chlorine are dosed into the water to form monochloramine. As chloraminated water travels through a water supply system, a gradual decay of chloramine occurs by auto-decomposition (Eq. 6.1) or reaction with natural organic matter (NOM) as in Eq. 6.2, and ammonia is released (Vikesland, Ozekin & Valentine 1998).

$$3NH_2Cl \longrightarrow N_2 + NH_3 + 3Cl^- + 3H^+ \tag{Eq. 6.1}$$

$$NOM + NH_2Cl \longrightarrow NOM_{ox} + NH_4^+ + Cl^- \tag{Eq. 6.2}$$

$$NH_2Cl + NO_2^- + H_2O \longrightarrow NO_3^- + NH_4^+ + Cl^- \hspace{2cm} \text{(Eq. 6.3)}$$

A sufficient increase in free ammonia (food) and decrease of monochloramine (disinfectant) provides suitable conditions for nitrification and proliferation of ammonia oxidizing micro-organisms (oxidise ammonia to nitrite) and subsequent nitrite oxidising microbes (oxidise nitrite to nitrate) (Regan, John M, Harrington & Noguera 2002). In chloraminated water distribution systems, partial nitrification (ammonia oxidation to nitrite) occurs, resulting in high nitrite levels in the system. Nitrite reacts with chloramine (Eq. 6.3) reducing it to ammonia. Since high nitrite level is always associated with a sudden drop of chloramine residuals, nitrification is traditionally thought to be the only cause of chloramine decay. As a result of this, research and operational control has mainly been focused on preventing or controlling it.

Questioning this traditional belief, Sathasivan, Fisher and Kastl (2005) developed the microbial decay factor method to measure the microbial and chemical components of chloramine decay. By measuring the decay coefficients as the chloramine gradually decayed in bulk water samples collected from a distribution system, Sathasivan, Fisher and Tam (2008) identified three distinct phases: the first was controlled only by chemical decay; the second contained significant microbial decay but nitrification was minimal (nitrite <0.01 mg-N$\cdot$L^{-1}); the third was indicated by high nitrification rates and a sudden drop in chloramine levels. They termed the second and third phases as mild and severe nitrification. On further analysing the samples from severely nitrifying samples, soluble microbial products (SMP), especially proteins of 30–50 kDa were found responsible for catalytic acceleration of chloramine decay in filtered, severely nitrifying samples and the decay was caused by acceleration of auto-decomposition (Eq. 6.1) and nitrite oxidation (Eq. 6.2) (Herath, Torres & Sathasivan 2018; Krishna, Sathasivan & Sarker 2012; Sathasivan & Bal Krishna 2012). Hereafter, the proteins accelerating the decay of chloramine are termed chloramine decaying proteins (CDP). Krishna, Sathasivan and Sarker (2012) and Sathasivan, Fisher and Kastl (2010) reported that the impact of CDP can be eliminated by silver (50 µg-Ag$\cdot$L^{-1}). The presence of CDP in filtered samples increased the total ammoniacal nitrogen (TAN) loss and expedited nitrite oxidation. Therefore, Herath, Torres and Sathasivan (2018) used these parameters as indicators of the concentration of CDP.

In order to maintain the recommended level of chloramine in the distribution system, it is essential to understand what factors affect the production of CDP. In many cases, impact

typical of CDP was noted in severely nitrifying samples. Assuming that the impact could arise from nitrification and that nitrification could be inhibited by high organic carbon content, Herath, Torres and Sathasivan (2018) operated reactor sets with varying NOM levels. The collected samples from severely nitrified reactors were subjected to re-chloramination. They concluded that the maximum impact of CDP in filtered water could be seen in water with the lowest DOC (<0.1 mg-C·L^{-1}). In unfiltered samples, however, the NOM levels did not have any impact. This raises the question whether that was due to the CDP associated with the biomass or due to the production of additional CDP by the microbes present in the biomass of unfiltered samples upon re-chloramination.

The possibility of additional production is hinted at in various findings. (Mir, Morato & Ribas 1997) explained that increased resistance to chlorine could be due to the synthesis of unique proteins or to the aggregation of bacteria. (Ridgway & Olson 1982) showed that biofilm attached bacterial colonies survived well in highly chlorinated (>1 mg·L^{-1}) environments by covering their body with extracellular mucopolysaccharide or glycoprotein polymers. Combining these with the results of other experiments, it was proven that the surface of the attached bacteria/biofilm provided the greatest increase in disinfection resistance. Other than disinfection, starvation or oxygenation also increased the resistance to chlorine in certain bacterial strains such as *Escherichia coli* (Saby, Leroy & Block 1999). EPS production is substantially enhanced when the microbial community is subject to stressful culture conditions (Liu, Y-Q, Liu & Tay 2004). Varin et al. (2012) detected bacterial adaptations such as EPS production, synthesis of cold shock proteins and membrane modification as a response to the environmental stress. Similarly, More et al. (2014) reported that microbes may improve function and survival under harsh conditions by enhancing their local microenvironment by producing EPS.

In a water distribution system, chloramine is not only added before water enters the system, but also after a certain time has elapsed to maintain certain a minimum chloramine residual. This is done to prevent nitrification from occurring by combination of the released free ammonia with added chlorine, or to recover from the status of severe nitrification. Because chloramine is a stressor, the CDP could be produced or released as a response. However, no literature so far has referred to such a phenomenon.

In this paper, experiments were conducted to identify whether the production or presence of CDP was a stress response to monochloramine dosing in a laboratory scale reactor system.

Two reactors were operated by adding inorganic substrates to Milli-Q water where the only variable was whether chloramine (Chloraminated reactor) or ammonia (Ammoniated reactor) was added. Milli-Q water was added to both reactors to maximize CDP production (Herath, Torres & Sathasivan 2018). The reactors were seeded from another laboratory scale reactor system (of five reactors connected in series) operated with the feed from a local water treatment plant (Herath, Torres & Sathasivan 2018). The seed was collected from a reactor undergoing severely nitrifying conditions. The impact of chloramine/ammonia on CDP production and release in the waters drawn from the chloraminated/ammoniated reactor was analysed. Overall fluctuations of nitrogenous species (ammonia, nitrite, and nitrate) and total chlorine (TCl) in the reactors and during chloramine decay tests are reported herein. To confirm that the CDP was only produced and released by micro-organisms living in the chloraminated reactor, short-term chloramine stress was applied and the results are reported.

6.2 Materials and methods

6.2.1 Reactor operation

Chloraminated and ammoniated reactors were operated as described in Chapter 3, section 3.4 in a water bath with a temperature of 20–23 °C. Both reactors were continuously operated during the experimental period.

6.2.2 Batch rechloramination tests

Samples from the chloraminated and ammoniated reactors were collected intermittently over a period of eight months. A part of each sample was filtered through a 0.22 µm sterilised membrane (non-pyrogenic, Millipore Express® polyether sulfone) attached to sterilised 33 mm syringe-driven filter units [housing limit 150 psi (10 bars), MILLEX®-GP, MILLIPORE IRELAND, Ltd.] for the removal of microbes. Unfiltered and filtered samples were named according to the reactor from which the sample was collected: unfiltered and filtered samples prepared from ammoniated (and chloraminated) reactors were named RA unfiltered and RA filtered (or RC unfiltered and RC filtered), respectively.

For the chloramine decay test, 200 mL of sample was placed in a 500 mL PET bottle. The bottles were pre-washed by topping up with a concentrated chlorine solution (20 mg-Cl_2.L^{-1}), left overnight for disinfection. Afterwards, the bottles were thoroughly washed with Milli-Q water prior to sample addition. The initial chemical composition such as chloramine residual (maintaining Cl-to-TAN ratio of 4:1) and pH were adjusted in each sample using respective stock chemical solutions. The adjusted chemical parameters were: total chlorine (3.35 mg-Cl_2.L^{-1}), ammonia (0.8 mg-N.L^{-1}) and pH 7.9 – 8.1. If there was nitrite in any of the samples collected from a reactor (for example, RC), the same nitrite levels were maintained in the other reactor (in this case, RA) for the purpose of comparison. At the time of adding chloramine (ammonia + chlorine) to the sample, the same dose was applied to Milli-Q water (and measured) to ensure that the desired dose was applied. Samples were incubated at 20 °C, and for each sample, total chlorine, ammonia, nitrite and NOx were monitored over time (for approximately 6 days), depending on the decay rate. All experiments were done in duplicate.

6.2.3 Short term chloramine stress

Biomass from each reactor was concentrated by filtering through 0.22 μm filter papers (Nitrocellulose membrane, GSWP03700, Merck Millipore, Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland). The filter paper holding the microorganisms was carefully removed using a forceps and added separately to 1 L of nutrient solution. The sample bottles were sonicated using an ultrasonic sonicator (50 Hz, FXP14M, Unisonics-Australia) for about 10 min to homogenize the collected microorganisms in the feed water; then the contents were divided into 4 sub-samples (250 mL each). The sub-samples were subjected to different conditions: 1.5 mg.L^{-1} chloramine stress (Cl:NH_3 was 4:1), only ammonia (0.375 mg-N.L^{-1}), silver (100 μg-Ag.L^{-1}) and nothing as control (i.e., nutrient solution only). A short-term chloramine shock (1.5 mg.L^{-1} initial dose) was applied and the solution incubated for over 1.5 h at 20 °C. All sub-samples were filtered through 0.22 μm filter paper to remove micro-organisms. A 200 mL portion of a filtered water sample from each sub-sample was separately collected into pre-cleaned PET bottles to carry out the chloramine decay test. Depending on the origin of the sub-samples (Chloraminated or Ammoniated reactor) they were named SC or SA. Experimental flow chat for applying short term chloramine stress is presented in Fig. 6.1.
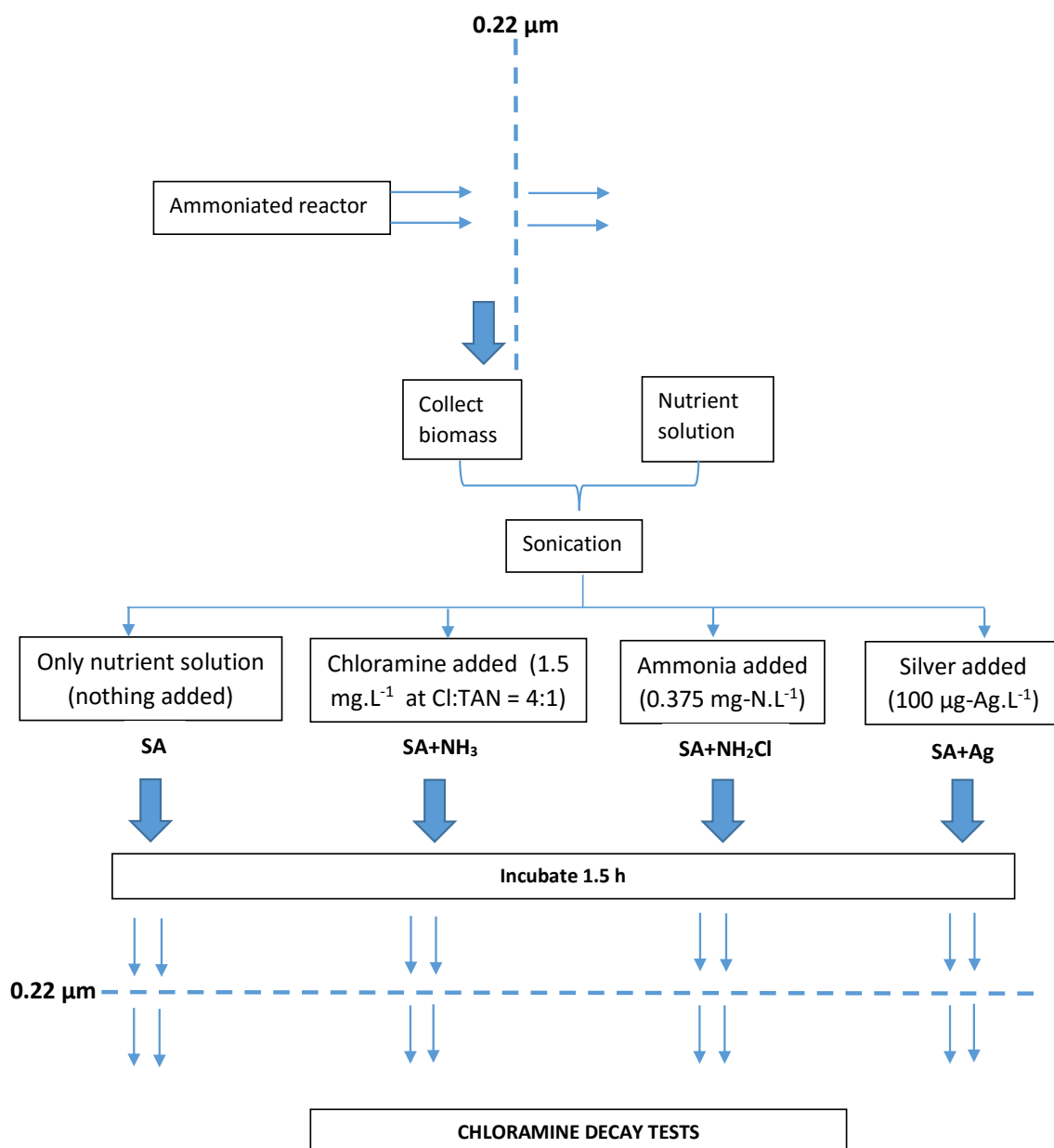
Figure 6.1: Flow chart for the application of short term chloramine stress for ammoniated reactor water. A similar procedure was implemented for the chloraminated reactor water, but the subsamples were named SC instead of SA.

6.3 Results and discussion

6.3.1 Operation of the reactors
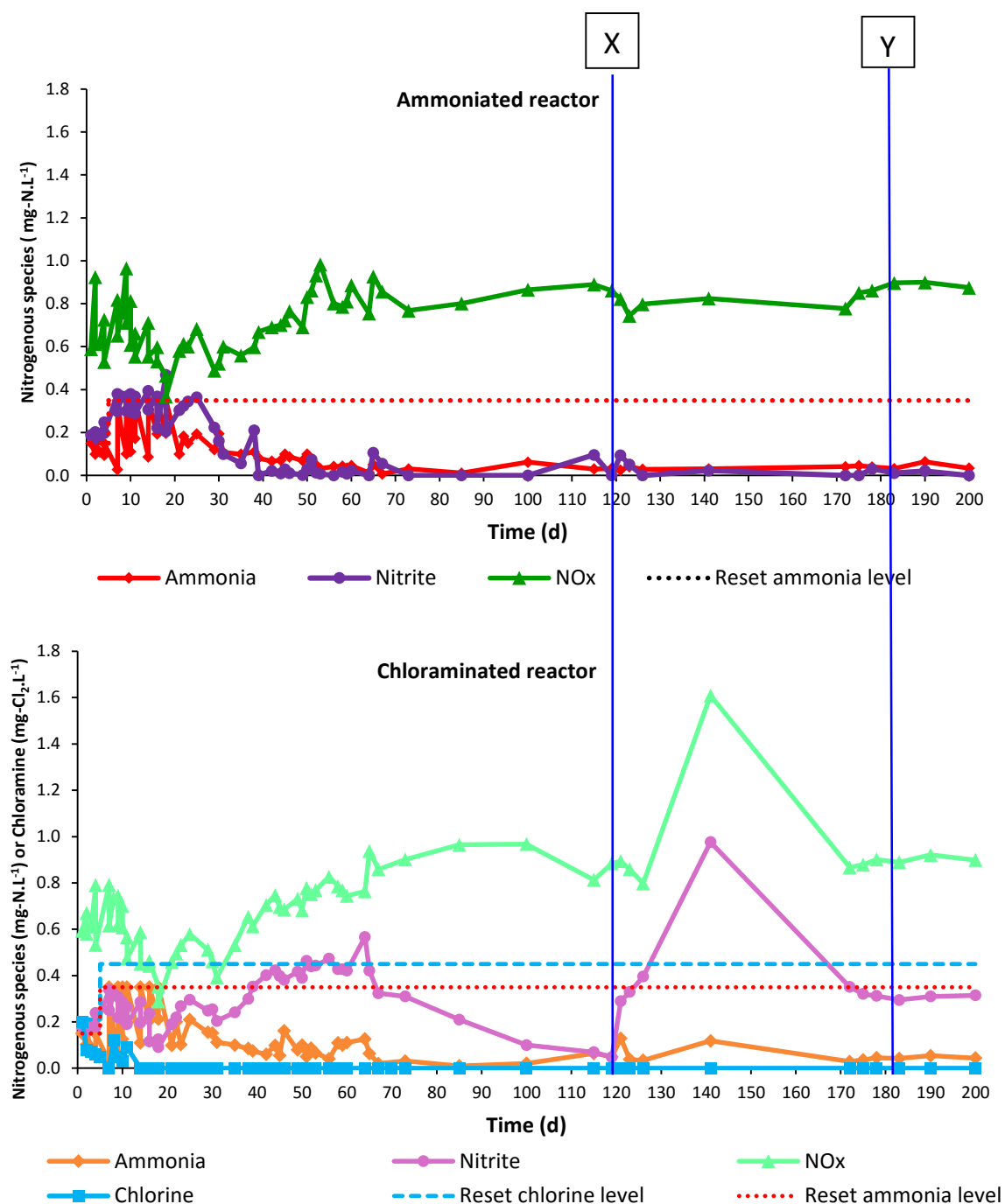
Figure 6.2: Changes in ammoniated and chloraminated reactors during the operational period. At the time of feeding, ammonia and chlorine concentrations were reset to the levels indicated. Profiles marked in solid line represent the concentrations just before feeding.

Within the first 35 days of the operational period, both ammoniated and chloraminated reactors had similar ammonia decay profiles. After feeding, the reset ammonia level (0.35 mg-N.L^{-1}) dropped to ≈ 0.20 mg-N.L^{-1} within a day in both reactors. At the time of adding nutrient solution, the nitrite level dropped slightly due to dilution (not shown); however, the level was restored on the next day (Fig. 6.2). Similar behaviour was observed by NOx levels and the results displayed ongoing partial nitrification in both the ammoniated and chloraminated reactors.

Significant behavioural changes in the ammoniated and chloraminated reactors were observed after the first 35 days. The ammoniated reactor shifted from partial to complete nitrification resulting in nil nitrite levels and increased NOx levels (≈ 0.559 mg-N.L^{-1} to ≈ 0.89 mg-N.L^{-1}). However, the chloraminated reactor indicated partial nitrification with high levels of nitrite (≈ 0.6 mg-N.L^{-1}), with even complete nitrification at times. As a result of the high nitrite level (and possibly CDP) observed in the chloraminated reactor, the chloramine level (from the reset level of 0.45 mg-Cl$_2$.L^{-1}) dropped to as low as 0 mg-Cl$_2$.L^{-1} within a day. Out of many batch tests, two typical results are discussed below to demonstrate the water quality under partial (point Y) and complete (point X) nitrifying conditions and how much soluble CDP was present.

On Day 119 (X in Fig. 6.2), the conditions in both reactors were completely nitrifying. Hence, the nitrite concentration was very low (less than 0.096 mg-N.L^{-1}) as almost all nitrite was converted into nitrate. A high level of NOx ($0.891 – 0.897$ mg-N.L^{-1}) and a very high loss of ammonia, demonstrated nitrifying microbial activity in both reactors. Although the pH was adjusted at each feeding time, it dropped from 8.00 ± 0.10 to $7.62 – 7.71$ by the time of sampling (Table 1). This further confirmed the presence of nitrifying bacterial activity in both reactors (Shammas 1986). The only sample difference observed was a high nitrite level (0.295 mg-N.L^{-1}) in the chloraminated reactor on Day 183 (Y in Fig. 6.2).

6.3.2 Batch re-chloramination test in filtered and unfiltered samples

(a)

(b)

(c)

(d)
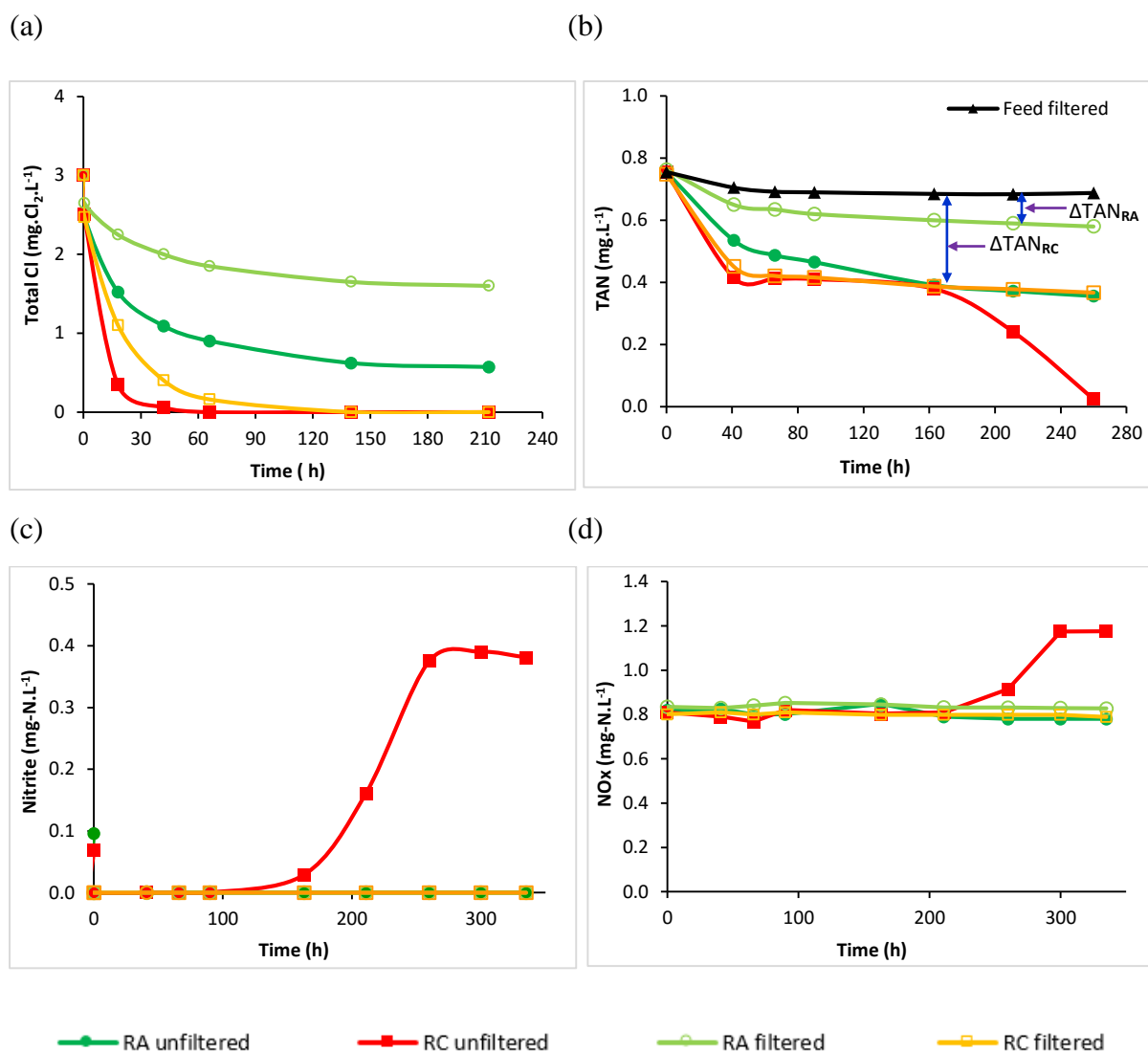
Figure 6.3: Total chlorine (a) and nitrogenous species –TAN (b), nitrite (c) and NOx (d) decay profiles of water samples collected from ammoniated (RA) and chloraminated (RC) reactor at point X in Fig 6.1

Because the initial conditions were adjusted to be the same (Section 6.2.2), the conditions among the samples collected can be compared at each point. In general, chloramine decayed much faster in unfiltered RC samples than in unfiltered RA samples at both X and Y for the same initial conditions (Figs 6.3 and 6.4). TAN reduction and nitrite production observed in the RC unfiltered samples were significantly higher than those in the RA unfiltered samples (Figs 6.3b and c).

To the ammoniated reactor, only ammonia was added (no chlorine); therefore, the microbial community living in this reactor was not acquainted with chloramine. Therefore, high chloramine concentration (3 mg-Cl$_2$.L^{-1}) and sustained high chloramine (>1.1 mg-Cl$_2$.L^{-1} for 42 h) in the samples collected from the RA reactor could have been toxic to the existing microbes and ensured no nitrification and slower chloramine decay. However, nitrifying microorganisms in the chloraminated reactor (RC) fed with chloramine-containing feed water, recovered from the shock of the high chloramine concentration, and were capable of producing nitrite and decaying the added chloramine significantly. The chloramine disinfection (>1.1 mg-Cl$_2$.L^{-1} for 10 h) in the chloraminated reactor (RC) samples was lower than that in RA due to faster chloramine decay. These results demonstrate that nitrifying microbes grown in a non-chloraminated environment do not survive a high chloramine dose; and that those grown in a chloraminated environment do survive, possibly by quickly decaying chloramine using CDP, as established previously (Herath, Torres & Sathasivan 2018).

On the other hand, the unfiltered samples showed much faster chloramine decay than that in the filtered samples from both ammoniated and chloraminated reactors, at points X and Y (Figs 6.3a and 6.4a) There could be three possible reasons for this: the presence of biomass-associated CDP, the additional production or liberation of CDP after the addition of chloramine as a shock response or to other mechanisms of microbial chloramine decay (Herath, Torres & Sathasivan 2018; Sathasivan, Fisher & Tam 2008). If the fast decay of unfiltered samples occurred as a shock response to chloramine, the long-term behaviour of the reactors and the shock response on the reactor biomass has to be investigated. Therefore, the next two sections address the conditions in filtered water samples during the operational period and how the reactor biomass responded to the stress (chloramine addition).

(a)

(b)

(c)

(d)
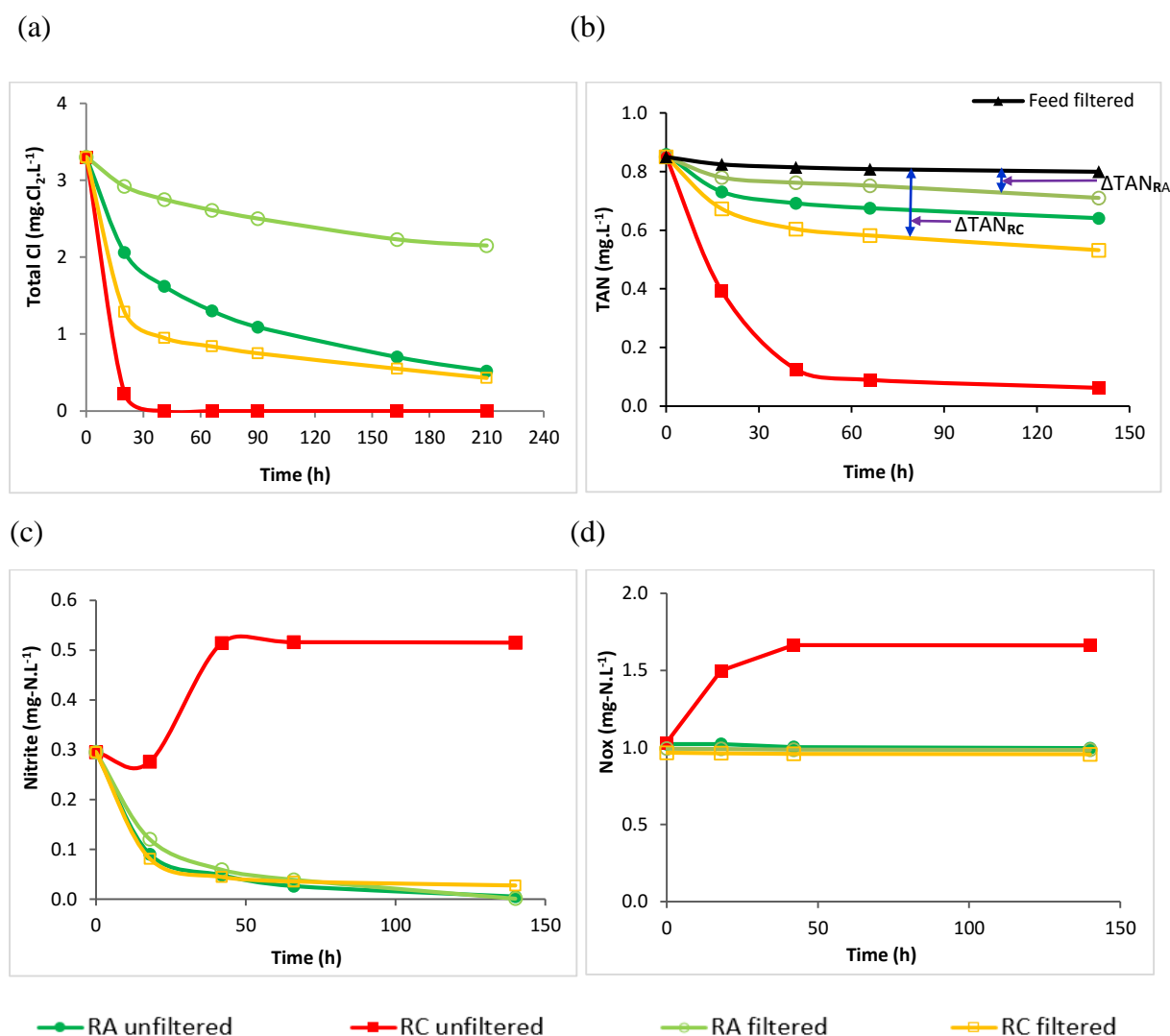
Figure 6.4: Total chlorine (a) and nitrogenous species: TAN (b), nitrite (c) and NOx (d) decay profiles of water collected from ammoniated (RA) and chloraminated (RC) reactor at point Y in Fig 6.1

6.3.3 Concentration of the soluble CDP in filtered water samples at X and Y

In filtered samples containing soluble CDP, it has been shown that the chloramine decay is associated with increased reduction rates of nitrite and total ammoniacal nitrogen (TAN). This is in contrast with that decay in feed water that has not been subjected to nitrification and with the decay in filtered severely nitrifying samples dosed with silver to inhibit the activity of CDP (Fig. B.1 and B.2). The impact of CDP, therefore, can be measured using the maximum difference in TAN (ΔTAN) values between two filtered samples, one containing and one not containing CDP (or CDP inhibited). Therefore, (Herath, Torres & Sathasivan 2018) suggested

that ΔTAN and/or ΔNO_2-N could be used as a surrogate measures of CDP in the absence of a suitable measurement method.

More than 71 – 87 % of the dosed chloramine was lost within the first 40 h of incubation in the filtered RC sample, while in the filtered RA sample only 17 – 33 % of the chloramine was lost. TAN results (Fig. 6.4b) in the filtered samples corresponded with chlorine decay results. Within the first 40 h of the incubation period, RC filtered samples demonstrated a sudden drop in TAN, corresponding to the chloramine decay results. After this initial drop, a negligible drop in TAN was observed during the rest of the experimental period. This initial sudden drop was much smaller (Fig. 6.4b) in the RA samples. Moreover, none of the filtered samples indicated the production of nitrite or NOx as all those samples were free from nitrifying bacteria (6.3d and 6.4d). As there was no nitrite present in the samples collected at point X, only the ΔTAN values were considered to confirm the existence of CDP. The RC filtered samples recorded higher ΔTAN values and thus higher concentrations of CDP (Table 6.1). All this evidence indicates a greater amount of CDP in RC unfiltered, than in RA filtered samples.

Table 6.1: ΔTAN values calculated for filtered samples collected at points X and Y

Sample	ΔTAN (mg.L^{-1})	
	Point X	Point Y
RC filtered	0.298±0.028	0.227±0.021
RA filtered	0.085±0.031	0.057±0.026

This observation and our previous findings (Herath, Torres & Sathasivan 2018; Krishna, Sathasivan & Garbin 2013; Krishna, Sathasivan & Sarker 2012) corroborate other reported outcomes of protein production as a disinfectant resistance response. The only difference is that the previous researchers reported the production of EPS, but did not report accelerated decay of the disinfectant. Flemming and Wingender (2010) showed proteins and polysaccharides in EPS which served as a protective barrier and which conferred resistance to disinfectants and antibiotics. More EPS with higher protein content were produced by antibiotic-resistant bacteria arising from the effect of antibiotics such as sulfadiazine and ciprofloxacin (Wang, Haibo et al. 2018). Chapman (2003) reported a possible survival

mechanism of *Mycobacteria* in chloraminated water as being the production of EPS to protect cells from direct exposure to disinfectants. The EPS produced by the members of *Methylobacterium* helps to reduce trihalomethene formation by forming aggregates with other bacteria in the drinking water (Tsagkari & Sloan 2018). Furthermore, when considering biofilms, enzymes present in the extracellular matrix may play a role in neutralizing toxic compounds (Habimana et al. 2011). This could also relate to our observation of the production or release of CDP due to chloramine toxicity stress. Therefore, it can be concluded that, in the presence of chloramine, microorganisms may produce and release proteins into the bulk water as a stress response. However, the decay of 17 – 33 % of the chloramine and minor ΔTAN noted in ammoniated reactor water samples could also be accountable for the CDP impact, which could possibly have been produced or released due to the presence of nutrient-stressed heterotrophic microorganisms.

6.3.4 ΔTAN profile over the period of operation in chloraminated and ammoniated reactors
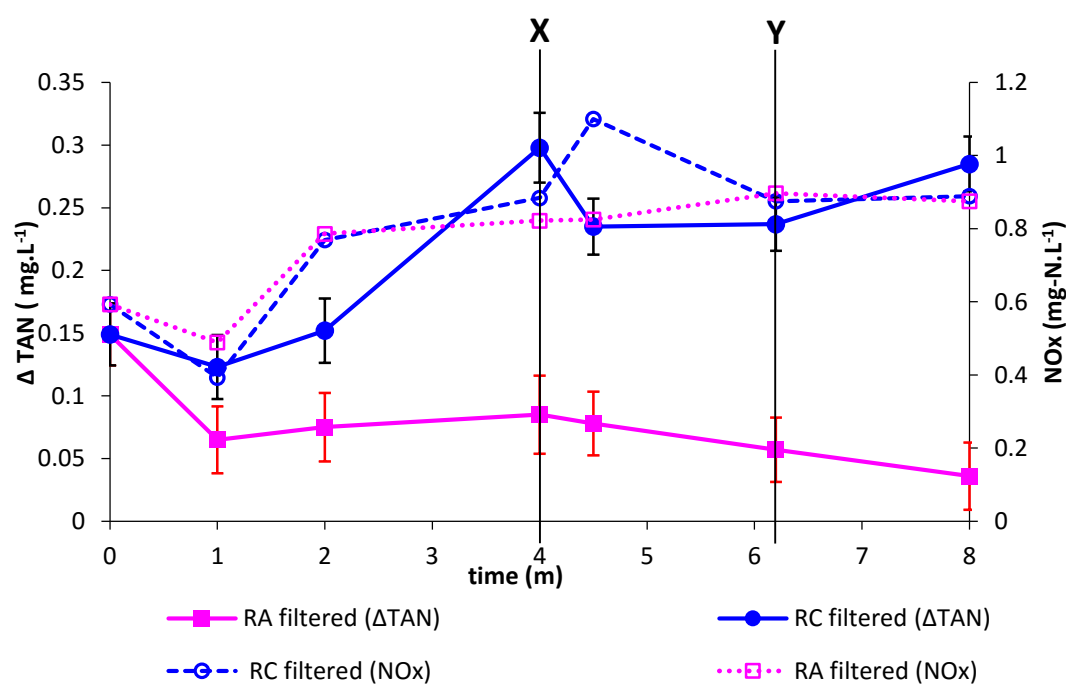
Figure 6.5: ΔTAN and NOx in RA filtered and RC filtered samples

At the beginning, there was no difference in ΔTAN (indicating the soluble CDP concentration) in the filtered samples from the chloraminated and ammoniated reactors as both reactors started with the same seed (Fig. 6.5). During the first month, the reactor microbes were incrementally adjusting to their new conditions. With time, the soluble CDP concentration started to increase in the chloraminated reactor. The maximum soluble CDP concentration was reached at 4 months and remained relatively constant afterwards. By this time, the microorganisms responsible for CDP production could have been at their maximum potential under the conditions observed. The low DOC level, pH and nutrients added for growth of the nitrifying microbes, could be at the optimum levels for their development. However, the soluble CDP concentration (ΔTAN) in the ammoniated reactor continuously decreased, although not to zero.

The ΔTAN behaviour in the chloraminated and ammoniated reactors could be explained by two possibilities. With time, production of CDP in the chloraminated reactor could have increased due to the application of frequent chloramine stress. On the other hand, microbes in the ammoniated reactor did not experience stress from the chloramine; hence the soluble CDP should gradually decrease towards zero. However, it showed a low, non-zero value. Additionally, the NOx variations in the chloraminated reactor displayed a strong correlation with the RC filtered ΔTAN (Fig. 6.5). The ΔTAN in the filtered RC samples increased alongside the NOx values of the chloraminated reactor; however, such behaviour was not observed in the ammoniated reactor. Because ΔTAN indicates the soluble CDP concentration in the bulk water, the presence, production or release of CDP could mainly be attributable to the presence of chloramine, in addition to the degree of nitrification.

Microbial community analysis of the chloraminated drinking water systems revealed the dominance of heterotrophic bacteria on several occasions, even with severe nitrification (Krishna, Sathasivan & Ginige 2013; Noguera et al. 2009; Williams et al. 2004). Nitrifiers are known to produce SMP from substrate metabolism and biomass decay (Barker & Stuckey 1999; Furumai & Rittmann 1992; Rittmann, Bruce E, Regan & Stahl 1994) . Experiments done by Kindaichi, Ito and Okabe (2004) and Okabe, Kindaichi and Ito (2005) gave evidence that SMP produced by nitrifiers provided the sole organic substrate for heterotrophic bacteria since there was no external organic carbon added in their experiments. Therefore, there is a high probability that the dominant heterotrophic bacterial population in the reactor lives under nutrient (carbon) limitation since the ammoniated and chloraminated reactors were maintained with no carbon added to the feed water (Milli-Q water with inorganic nutrients). This limited nutrient condition could promote EPS production by heterotrophic bacteria as a response to

stress from nutrient limitations (Flemming & Wingender 2010; Sutherland 2001). Therefore, without having enough evidence from such as pure culture experiments, CDP production/release cannot be assigned only to nitrifying micro-organisms: heterotrophic bacteria could also play an important role.

In summary, the results clearly demonstrate not only nitrification but also chloramine stress is needed to induce the production and release of CDP, and hence increase the soluble CDP. When the same community was exposed to non-chloraminated but nitrifying conditions, the soluble concentration of CDP was minimal.

6.3.5 Short term chloramine stress induces CDP production by the biomass in the chloraminated reactor

As confirmed above, the microbes living in a chloraminated environment release or produce CDP due to applied chloramine stress. Even though the ammoniated reactor was nitrified to the same or greater extent as the chloraminated reactor, CDP production was minimal. Therefore, the minimal CDP condition in the ammoniated reactor could be explained by two possibilities. One possibility would be that the microbes living in the ammoniated reactor could also produce CDP, although it was not released into the bulk water due to no stress condition. Another possibility would be that the CDP production did not occur there because no stress was applied to the bacteria occupying the reactor. Similarly, the bacteria in the chloraminated reactor, which did produce and release CDP, might have been able to release more CDP if additional chloramine stress had been applied. These possibilities are discussed with the results achieved in the following experiments conducted by harvesting bulk water biomass and subjecting them to short-term chloramine stress (1.5 mg.L^{-1} over 1.5 h).

(a)

(b)

SC   SC+NH3   SC+NH2Cl   SC+Ag

(c)

(d)

SA   SA+NH3   SA+NH2Cl   SA+Ag
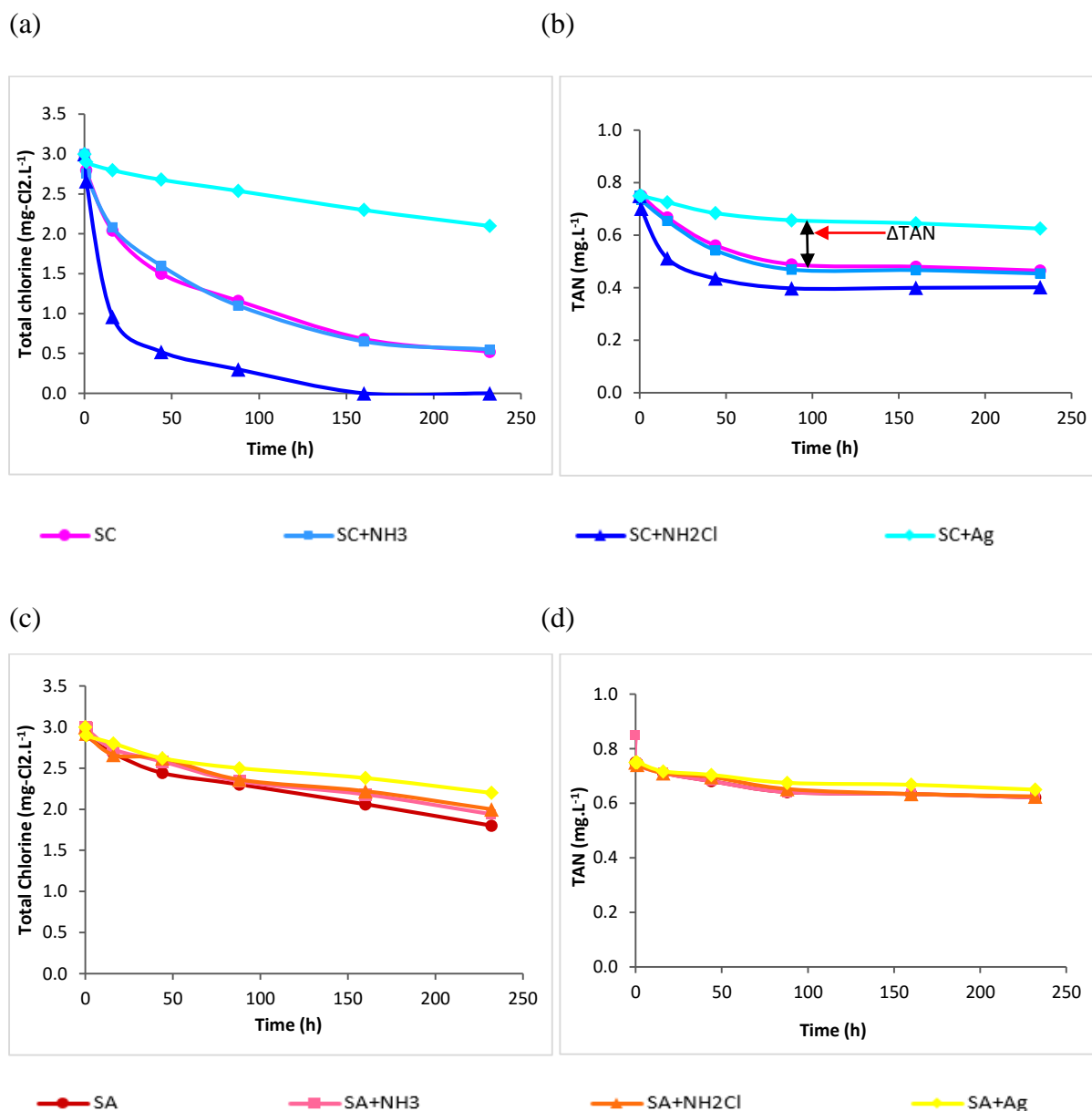
Figure 6.6: Total chlorine (a and c) and TAN (b and d) profiles of the filtered samples obtained from chloraminated and ammoniated reactor biomass incubated with nutrient solution (SC/SA), nutrient solution with ammonia (SC+NH$_3$ / SA+NH$_3$), nutrient solution with chloramine (SC+NH$_2$Cl / SA+NH$_2$Cl) and nutrient solution with silver (SC+Ag / SA+Ag). Nitrite is not present in the samples

To demonstrate the decay profiles with no CDP contribution for ΔTAN derivation, a sample from each reactor was incubated with silver. Krishna, Sathasivan and Sarker (2012) showed a dose of 50 µg-Ag.L^{-1} was sufficient to inactivate the CDP in a microbe-free (filtered) sample. In our experiment, 100 µg-Ag.L^{-1} was used as precaution to inactivate the CDP activity. Silver has been used as an effective water disinfectant for many decades (Kim, Jaeeun et al. 2004;

Russell & Hugo 1994). Among all the heavy metals, silver ions have the highest level of antimicrobial activity due to their strong bonding with the disulfide (S–S) and sulfhydryl (–SH) groups found in the proteins of microbial cell walls (Feng et al. 2000; Slawson et al. 1992). Therefore, in the presence of silver, normal metabolic processes are disrupted, leading to the death of microbial cells. Silver nanoparticles exert their anti-microbial effect through interaction with protein thiol groups (Elechiguerra et al. 2005; Morones et al. 2005; Nel 2005).

The samples consisting of the biomass extracted from the chloraminated reactor demonstrated much higher chloramine and TAN decay profiles than for the biomass from the ammoniated reactor. The silver augmented chloraminated or ammoniated samples (SC+Ag / SA+Ag) recorded the lowest chloramine loss due to the inhibition of CDP activity by silver (Krishna, Sathasivan & Sarker 2012). However, the SC sample incubated only with nutrient solution (not with ammonia or chloramine) also indicated the presence of CDP — possibly because the CDP was attached to the biomass and desorbed into the solution. The microbial sample incubated with ammonia (SC+NH$_3$) led to decay rates similar to that of the SC sample. Among these, the chloramine augmented SC showed the highest chloramine decay and commensurate additional TAN reduction, indicating additional production of CDP as a response to disinfectant (stress). The highest ΔTAN (0.259 mg.L^{-1}) was recorded in the SC+NH$_2$Cl sample while the SC and SC+NH$_3$ samples indicated 0.168 and 0.178 mg.L^{-1} of ΔTAN, respectively.
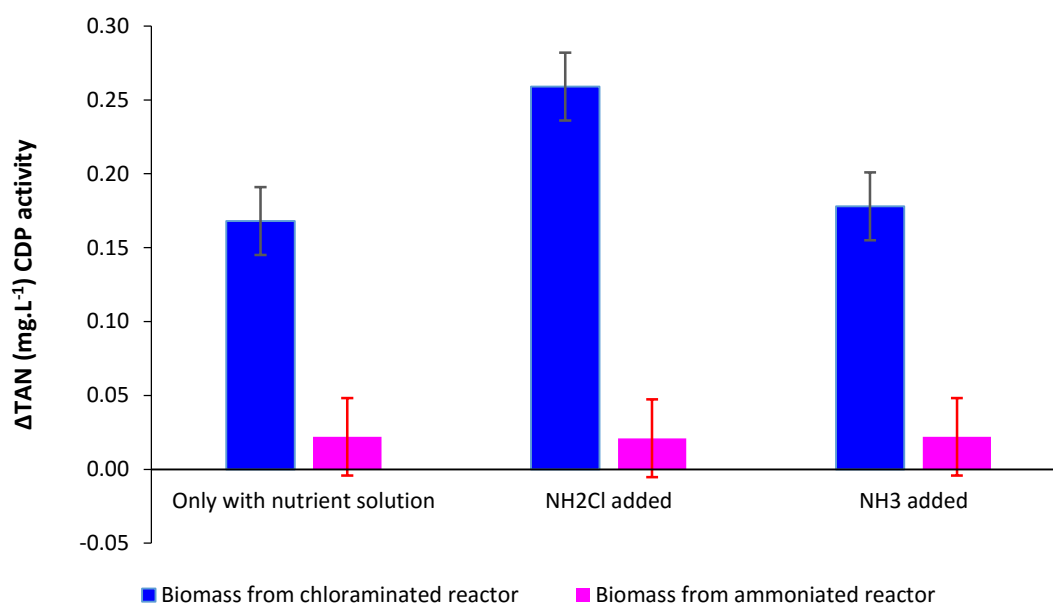
Figure 6.7: The production/release of CDP by microbial biomass collected from chloraminated and ammoniated reactor after the short term chloramine stress (nitrite or NOx were not present in the tested samples)

The chloramine and TAN decay profiles observed in samples prepared from the ammoniated reactor biomass indicated the same decay rates for all samples during the experimental period. The ΔTAN values for SA, SA+NH$_3$ and SA+NH$_2$Cl samples were 0.034, 0.034 and 0.023 mg.L^{-1} respectively. The resulting SA+Ag sample decay profiles were the same as the other ammoniated samples, indicating the absence of CDP activity. On the other hand, chloramine addition did not induce the production or release of CDP in the ammoniated samples (SA).

Such observation also questions the traditionally drawn conclusions, for example by some previous authors (Pressman et al. 2012; Sawade et al. 2016). Sawade et al. (2016) concluded that the microbial decay factors (F_m) in nitrifying and non-nitrifying samples were the same and that F_m was not a good indicator of the degree of nitrification. While the purpose of F_m was not to measure the degree of nitrification but to measure the degree of chloramine decay present in a sample collected from chloraminated system, applying chloramine to non-chloraminated microbes defeats the purpose. By applying chloramine at different Cl-to-TAN ratios to biofilm growing in a non-chloraminated environment, Pressman et al. (2012) showed that chloramine penetrates completely into deep biofilm layers, but also that inactivation is more effective with a higher Cl-to-TAN ratio. They further concluded that biofilm could be controlled by maintaining a high chlorine concentration. If the biofilm was grown in the chloraminated environment, applied chloramine could have been consumed by the CDP either already present in the biomass (biofilm) or by that produced during the exposure to chloramine, thereby limiting the effectiveness and penetration of chloramine. Therefore, at what concentration the biofilm could be controlled needs re-evaluation, although intuitively a high Cl-to-TAN ratio should provide better disinfection.

6.3.6 Implications for further research

It is essential to maintain a sufficient level of the disinfectant in drinking water distribution systems to prevent the regrowth or survival of waterborne pathogens. We have repeatedly and consistently shown with nitrification (Sathasivan, Fisher & Tam 2008) as well as without nitrification (Herath, Sathasivan & Lam 2015) that chloramine can decay faster when the chlorine concentration is lower (Sathasivan, Fisher & Kastl 2010). Various authors have observed highly accelerated chloramine decay after nitrification and difficulty in recovering chloramine residuals by rechloramination (Harrington et al. 2002; Kirmeyer 2004; Skadsen 1993; Wolfe et al. 1988), but all authors so far explained the phenomena in relation to the nitrite

present in the sample, and more recently, in relation to co-metabolism. In proving co-metabolism, the authors used *Nitrosomonas europea* grown under non-chloraminated condition to test chloramine decay at a very low chloramine concentration. Whether co-metabolism exists in a chloraminated system is questionable for two reasons: *Nitrosomonas europea* is not indigenous to chloraminated water supply systems (Regan, John M, Harrington & Noguera 2002) and growing in a non-chloraminated environment may not motivate the development of appropriate survival mechanisms.

Under severely nitrifying conditions, the CDPs are present in the bulk water in soluble form (Herath, Torres & Sathasivan 2018; Krishna, Sathasivan & Sarker 2012). Now, we have shown that CDP could also be present in solid form and could desorb back into bulk water, and that CDP is produced as a stress response upon the application of chloramine. We also showed that chloramine decay by the CDP is catalytic in nature meaning the rechloramination does not reduce the concentration of CDP (Krishna, Sathasivan & Sarker 2012), but excess (free) chlorination could only be a better strategy for filtered samples (Krishna, Sathasivan & Kastl 2014). In this work, we also have shown that soluble CDP could be accountable for up to 70 – 87 % (Fig. 6.3 and 6.4) of chloramine demand within 40 h, implying that the chloramine residual can be recovered only in the absence of CDP and CDP producing microbes. This is impossible in a chloraminated environment, which induces the production of CDP.

Under severely nitrifying conditions, nitrifiers are a minority (1–2%) as opposed to heterotrophs (Krishna, Sathasivan & Ginige 2013). Therefore, it is unknown which microbes actually produce the CDP under severely nitrifying conditions. Therefore, future research should concentrate on identifying the microbes responsible for producing CDP.

6.4 Conclusion

It is important to maintain sufficient chloramine residuals for the maintenance of disinfection. How microbes in chloraminated nitrifying water destroy the chloramine and survive by producing chloramine decaying protein (CDP) was studied. Auto-decomposition and/or nitrite oxidation were known to induce additionally by CDP and accelerates chloramine decay. In a filtered sample with a very low nitrite concentration, additional total ammoniacal nitrogen (ΔTAN) loss in a CDP-containing sample was used to measure additional auto-decomposition and thus to indicate the CDP concentration. Two laboratory scale reactors were fed

continuously to maintain either 0.45 mg-Cl_2.L^{-1} chloramine at the chlorine-to-nitrogen molar ratio of 1:1.4 (chloraminated) or 0.1 mg.L^{-1} NH_3-N (ammoniated) at the time of dosing. Chloramine induced CDP production and release were further established by applying short term chloramine stress on suspended microbes concentrated from each reactor.

The conclusions drawn from these experiments are as follows:

- NOx concentration increased in both reactors, but the soluble CDP concentration behaved differently. The soluble CDP concentration increased in the chloraminated reactor but decreased in the ammoniated reactor.

- Application of 1.5 mg·L^{-1} chloramine for 1.5 h induced the production or release of CDP from the biomass collected from the chloraminated reactor, but not from the biomass collected from the ammoniated reactor.

- Microbes in the bulk water from the ammoniated reactor could not bounce back from the introduction of high dose (3–3.4 mg-Cl_2.L^{-1}): possibly because chloramine stayed high over a longer period, or possibly due to the absence of a defense mechanism for producing CDP.

- Microbes in the bulk water from the chloraminated reactor bounced back from the introduction of a high dose of chloramine (3–3.4 mg-Cl_2.L^{-1}) by quickly destroying the chloramine (within 30 h). This was possibly due to the defence mechanism that involves the presence of CDP in soluble and insoluble forms, and to the ability to produce additional CDP as a stress response.

Chapter 7

Identification of bacteria decaying chloramine as a stress response

7.1 Introduction

Chloramine has been demonstrated to produce lower concentrations of disinfection by-products (DBPs) such as trihalomethanes and haloacetic acid and it has been noted that chloramine has less reactive properties compared to free chlorine (Hua & Reckhow 2008; Regan, John M, Harrington & Noguera 2002; Richardson et al. 2000). Therefore, many of the drinking water utilities in Australia and USA have implemented chloramination as a means for secondary disinfection (Moradi et al. 2017; Sarker, Sathasivan & Rittmann 2015). However, due to the excess ammonia produced from chloramine formation and the additional ammonia being released from chloramine decay, nitrifying micro-organisms colonise in the chloraminated drinking water systems and therefore, start accelerating chloramine decay.

Nitrification is oxidation of ammonia by two groups of chemolithotrophic bacteria, ammonia oxidizers and nitrite oxidizers. Several authors (Cunliffe 1991); Ike, Wolfe and Means (1988); (Lieu, Wolfe & Means 1993; Lipponen, Suutari & Martikainen 2002; Odell et al. 1996; Skadsen 1993; Wilczak et al. 1996; Wolfe et al. 1988) have identified that the nitrification in chloraminated drinking water systems can occur even with high doses of chloramine. Identification of nitrifying bacteria in chloraminated drinking water systems have been

conducted by many researchers (Krishna, Sathasivan & Ginige 2013; Lipponen et al. 2004; Regan, John Michael 2001; Regan, John M et al. 2003; van der Wielen, Voost & van der Kooij 2009). According to Regan, John M et al. (2003) the most abundant ammonia oxidising bacterial (AOB) genus in chloraminated systems (pilot and full-scale) is *Nitrosomonas oligotropha* while no *Nitrosospira* is identified. Among nitrite oxidising bacteria (NOB), *Nitrospira* is the most common genus while *Nitrobactor* has also been found in few samples. The biofilm examination in chloraminated drinking water by Lipponen et al. (2004) confirmed *Nitrosomonas* was the most ubiquitous AOB genus.

Identification of nitrifying bacteria in chloraminated systems has not proven that the primary cause for accelerated chloramine decay in chloraminated systems is nitrification. Most recently Krishna, Sathasivan and Sarker (2012) reported that a foreign agent present in nitrified water accelerates the decay of chloramine at a higher degree when compared with nitrification related decay rates. They also provided enough evidence to prove that this chloramine decaying agent termed as Soluble Microbial Products (SMP) are protein/s. However, this SMP currently termed as CDP was confirmed as proteins (Herath, Torres & Sathasivan 2018). According to our experiments conducted by maintaining chloraminated and ammoniated reactors utilising severely nitrified water as the seed water, it has been proved that this CDP can only be observed in the presence of chloramine, but not when there is only ammonia present (Chapter 6). This is enough evidence to demonstrate that this CDP is produced or released due to the stress induced by chloramine present in the reactors/systems. It is a well-known fact that the extracellular polymeric substances (EPS) are produced (or enhance their production) by bacteria due to the harsh, stress conditions (Liu, Y-Q, Liu & Tay 2004; More et al. 2014; Varin et al. 2012). Therefore, this CDP can also be included in to EPS. However, the responsible bacteria for this production/ release of CDP have not yet been identified.

As CDP has always been seen in nitrified water samples collected from severe nitrification stages, it is most likely to be released/ produced by nitrifying micro-organisms. Introducing a term as chloramine decay index (C.D.I) to assess the chloramine decay occurrence in the chloraminated drinking water systems, Moradi et al. (2017) showed that the formation of nitrite/nitrate and/or SMP and/or the release of EPS during nitrification may contribute to the C.D.I. increase. Additionally, in the presence of chloramines AOB produces organic compounds (Pan & Umbreit 1972; Rittmann, Bruce E, Regan & Stahl 1994). Rittmann, Bruce E, Regan and Stahl (1994) reported that NOB also produces soluble organic compounds which can contribute to the heterotrophic bacterial growth. Nevertheless, there are several evidence

to prove heterotrophic bacteria produces EPS due to various reasons. *Mycobacteria* in chloraminated-treated water produces EPS as they can protect cells from direct exposure to disinfectants (Chapman 2003). EPS produced by *Methylobacterium* form aggregates with other bacteria and reduce the THM content in the drinking water (Tsagkari & Sloan 2018).

Herath, Torres and Sathasivan (2018) demonstrated the impact of CDP present in nitrified water samples generally decreased as the dissolved organic carbon (DOC) of the feed water increased. Alternatively, microbes other than nitrifiers could equally decay chloramine (Herath, Sathasivan & Lam 2015). Herath, Sathasivan and Lam (2015) also demonstrated the onset of nitrification with low DOC containing water having maximum decay rate. Therefore, it is beneficial to be maintaining reactors with low DOC for the identification of CDP producing/releasing nitrifying bacterial community.

For chloraminated drinking water distribution systems, having a precise knowledge regarding chloramine decaying micro-organisms are critical to maintain recommended disinfection level in order to ensure safe delivery of drinking water to the customers. In the current study, the identity of bacteria presents in CDP containing severely nitrified water samples were examined by sequencing of 16S RNA. The compared samples were, seed water, ammoniated reactor water (without chloramine) and chloraminated reactor water. Decay characteristics of the same water samples were also identified together with the bacterial community identification.

7.2 Materials and Methods

7.2.1 Reactor operation

Ammoniated and chloraminated reactors were maintained/operated as described in Chapter 3 section 3.4. Seed water sample was collected from another lab scale reactor set, which was maintained as described in Chapter 3, section 3.3. To initiate nitrifying conditions in the ammoniated and chloraminated reactors, this seed water within the severely nitrified stage was used.

7.2.2 Sample preparation

Bulk water (2 L) from the severely nitrified reactor which was used to maintain chloraminated and ammoniated reactors were collected as the seed water from the sampling tap in to the sterile glass bottle. The sampling tap was kept open for 5 s to remove the water inside the pipe lines prior to bulk water collection. After 6 months of operational period, 2 L of bulk water samples from ammoniated and chloraminated reactors were also collected. Total chlorine (TCl), total ammoniacal nitrogen (TAN), DOC, pH, nitrite and nitrate were measured immediately after collecting the samples. Water samples (2 L) were filtered through a 0.22 μm filter paper (Nitrocellulose membrane, GSWP03700, Merck Millipore Ltd, Tullagreen, Carrigtwohill Co. Cork, IRL) to concentrate the biomass. According to the sample collected reactor, extracted samples were named as RA unfiltered, RC unfiltered and seed unfiltered for ammoniated, chloraminated and seed water collected reactors, respectively. Prepared filtered samples were named as 'filtered'.
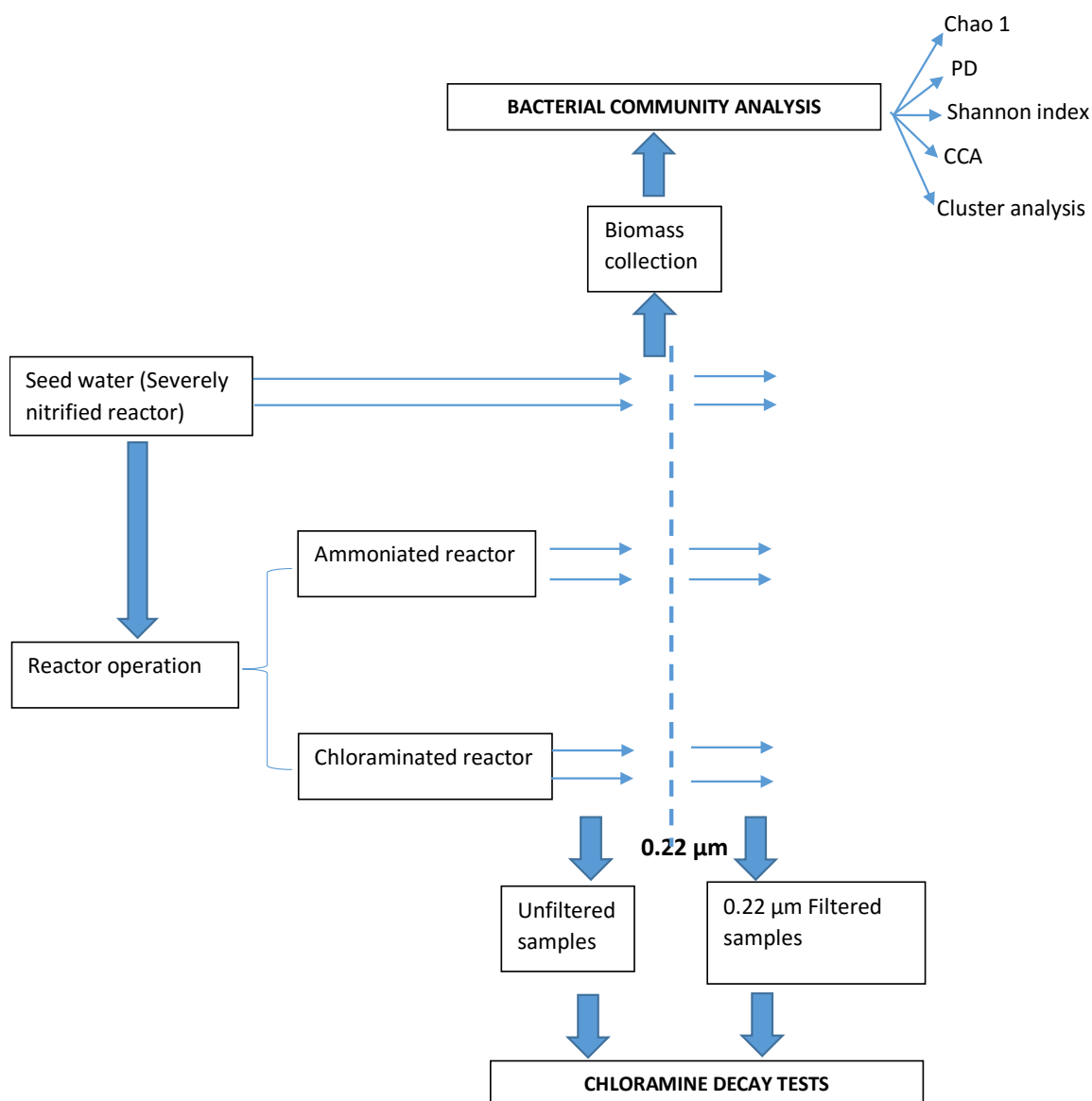
Figure 7.1: Detailed experimental plan

To perform chloramine decay tests, initial chemical compositions such as residual chloramine, nitrite, and pH, were adjusted in each sample using the respective stock chemical solutions. To achieve the expected chloramine concentration (3 mg-Cl_2.L^{-1}) in the water sample, ammonia was added (maintaining Cl- to- TAN mass ratio of 4:1) prior to chlorine addition. The nitrite level was adjusted depending on the concentration in samples at the time of sampling and the pH was adjusted to 7.9–8.1. The samples were incubated at 20 °C in a water bath while the experiment was in progress.

For each sample, the TCl residuals were periodically monitored and the decay rate was estimated. When chlorine was added to the sample, the same dose was added to Milli-Q water, and chlorine concentration was measured to ensure that the correct dose was applied. All experiments were conducted in duplicate.

The ΔTAN and ΔNO$_2$-N explain the impact of CDP on auto-decomposition and nitrite oxidation, respectively. Calculations were carried out considering the no nitrified feed water sample (described in section 2.3, Herath at al, 2018) behaviours as the baseline. The impact of CDP, therefore, can be measured using the maximum ΔTAN and ΔNO$_2$-N values, which are used as a substitute.

Parallel to the decay tests, DNA was then extracted from the concentrated biomass (~0.5 mL) using the QIAamp® DNA mini kit (Cat. No. 51304, QIAGEN GmbH, D-40724 Hilden) following the manufacturer's instructions. Before the samples were being analysed further, DNA purity was measured using Nano Drop 2000c spectrophotometer. A ratio of absorbance at 260 nm and 280 nm (A260/230) greater than 1.8 are typically considered suitable for analysis. This analysis was performed with 1 µL of DNA sample. The extracted samples were kept in a freezer (< -20 ºC) until outsourced for sequencing. Detailed experimental plan is presented in Fig 7.1.

7.2.3 16S RNA sequencing

The DNA extractions were then stored at -20 °C prior to shipment to MR DNA (Molecular research LP, Texas, USA) for Illumina MiSeq sequencing. Shipment was at room temperature on stabilizing the DNA using DNA stable Plus (Biometrica, Diagnostic Technology). Sequencing was carried out using MiSeq Illumina sequencing platform. In brief, HotStarTaq Plus Master Mix Kit (Qiagen, USA) was used for a single-step 28 cycle Polymerase Chain Reaction (PCR) amplification using 16S universal bacterial primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT). The thermocycler conditions used included an initial denaturing followed by 28 cycles of denaturation at 94 °C for 30 s; annealing at 53 °C for 40 s; and an elongation at 72 °C for 1 min; and a final elongation step at 72 °C for 5 min. After amplification, PCR products were checked in agarose gel (2%) to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their molecular

weight and DNA concentrations. Pooled samples were purified using calibrated Ampure XP beads. Then the pooled and purified PCR product was used to prepare Illumina DNA library. Sequencing was performed at MR DNA (www.mrdnalab.com) on a MiSeq following the manufacturer's guidelines.

7.2.4 Post sequencing process

Post sequencing process of data was carried out using Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al. 2010) software package (http://www.qiime.org). Maximum sequence length was set at 600 and fast, qual and mapping files were used as input for split_libraries.py script with default arguments to extract sequences relevant to this study. Then all sequences were groups into operational taxonomic units (OTUs) using pick_otus.py script in QIIME using USEARCH method (Edgar 2010). The OTUs were defined at 80% sequence similarity and minimum cluster size was 1. Then using the PyNAST default method a representative sequence from each OUT was selected and aligned against Greengenes imputed core reference alignment using align_seqs.py script (Caporaso et al. 2010). The gaps of the aligned sequence were removed using script filter_alignment.py. With default settings- Fast Tree phylogenetic tree and Ribosomal Database Project classifier and Greengenes OTUs data set- taxonomy assignment were constructed using the script make_phylogeny.py and script assign_taxonomy.py, respectively.

To analyse different aspects of microbial community following were derived using alpha_rarefaction. py script, assess the bacterial coverage- rarefaction curve, estimate species richness- Chao 1, phylogenetic coverage of OUT- phylogenetic diversity (PD), bacterial diversity- Shannon index and statistically estimate similarities between clone libraries- Jaccard index.

Canonical correspondence analysis (CCA) was used to show how environmental factors related to the bacterial communities in chloraminated, ammoniated and seed reactors. Matching correspondences between three reactors with cluster analysis using PAST software was performed using Bray-Curtis similarity index (based on 80% sequence similarity) and revealed the differences and similarities of bacterial communities among reactors.

7.3 Results and discussion

7.3.1 Characteristics of collected water samples

The chloraminated and ammoniated reactor water samples were analysed for chloramine decay characteristics and bacterial community compositions. DNA extraction and chloramine decay tests for seed water samples were performed at time of collection of water samples being used to feed/maintain the ammoniated and chloraminated reactors. The observed parameters of water samples tested are recorded in Table 7.1.

Ammoniated and chloraminated reactor water samples consisted of low DOC levels (0.1±0.08 mg-C.L^{-1}) as the reactors were maintained with feed water prepared using Milli-Q water and added nutrients. At the time of water collection for the decay tests and microbial community analysis, chloraminated water sample consisted of high nitrite levels (0.207 mg-N.L^{-1}) as the rectors were in partial nitrifying condition. With high level of NOx and no nitrite, the ammoniated reactor demonstrated complete nitrification. The chloraminated reactor was fed with chloramine while the ammoniated reactor was fed with only ammonia. Therefore, the ammoniated reactor water sample contained no total chlorine. In the same way, the chloraminated water sample demonstrated low TCl level (0.03 mg.L^{-1}) as a result of accelerated chloramine decay.

The DOC level of the seed reactor water was 4.5 mg-C.L^{-1} since this water collected from another reactor set. In the same was as the chloraminated reactor, seed water also established with partial nitrifying conditions having high nitrite levels (0.285 mg-N.L^{-1}) and recognized with low TCl (0.25 mg.L^{-1})

Additionally, the low TAN readings observed in all reactors indicated severely nitrified conditions as all TAN has been utilised. Furthermore, pH also dropped down to 7.61-7.77 from adjusted pH 8.0 in ammoniated and chloraminated reactors. Nitrification can reduce the pH of the water and the nitrite produced by nitrification can increase chloramine decay (Zheng et al. 2016). However, this accelerated chloramine decay along with severely nitrified conditions also accountable for the CDP impact (Herath, Torres & Sathasivan 2018; Krishna, Sathasivan & Sarker 2012).

Table 7.1: Parameters of the collected water samples

Parameters	Seed	Ammoniated	Chloraminated
Total Chlorine (mg-Cl$_2$.L^{-1})	0.25±0.03	0	0.03
TAN (mg.L^{-1})	0.105±0.005	0.031±0.005	0.042±0.005
Nitrite (mg-N.L^{-1})	0.207±0.003	0.011±0.003	0.285±0.003
pH	7.61—7.65	7.71—7.75	7.72—7.77
DOC (mg-C.L^{-1})	4.5±0.08	0.1±0.08	0.1±0.08

7.3.2 Batch rechloramination tests for reactor waters

In the unfiltered water samples, the Seed unfiltered and RC unfiltered demonstrated 92% and 98% of chloramine loss from the initial dose within first 40 h of incubation time (Fig. 7.2). However, the RA unfiltered water sample resulted only 66% of chloramine decay. Since all of the samples were in a severely nitrified condition, the accelerated decay could be resulting in all samples due to the nitrifying bacterial activity and the presence of CDP. The chemical parameters (increase of nitrite and drop of TAN) of collected water samples confirmed the active presence of nitrifying micro-organisms. If all samples consisted with CDP, the filtered samples should result in enhanced decay of chloramine. However, the decay results within the first 40 h of the incubation period were 74%, 85% and 36% for Seed, RC and RA filtered samples, respectively.
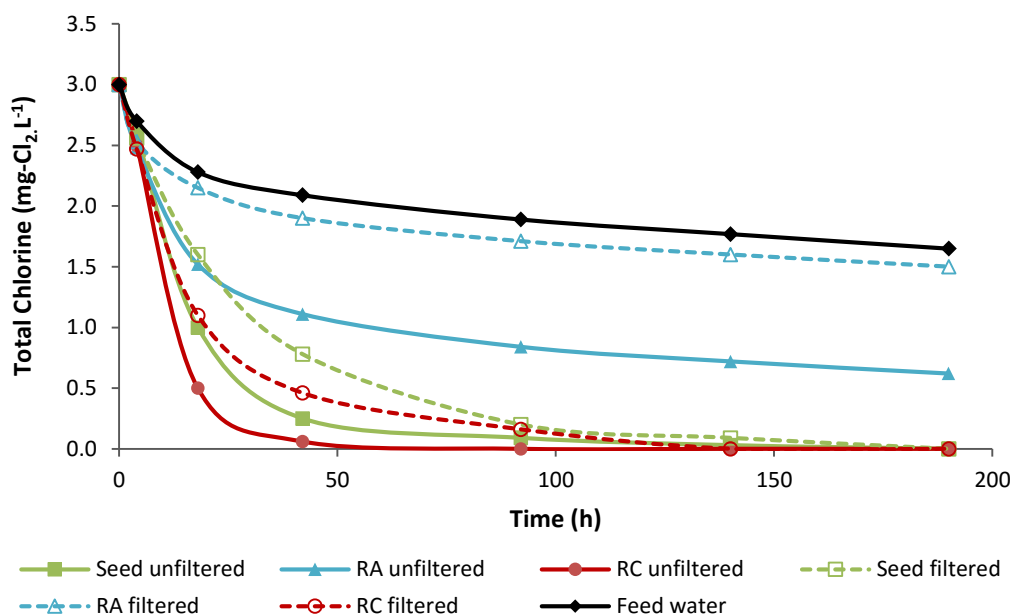
Figure 7.2: TCl decay profiles of the ammoniated (RA), Seed and chloraminated (RC) reactor bulk water. No nitrified Feed water behaviour used for the comparison of above nitrified samples.

Additionally, TAN and nitrite results (Figs 7.3a and 7.3b) demonstrated nitrifying bacterial activity in the Seed unfiltered and RC unfiltered samples. Both samples revealed a decrease of TAN levels along with an increase of nitrite levels. The rapid decline of TAN and the rapid incline of nitrite in RC unfiltered sample compared with the Feed unfiltered sample could be due to the increment of favorable conditions (low DOC, a supplement of nutrients) for nitrifying micro-organisms in the sample collected reactor. However, the RA unfiltered sample did not record any reduction of TAN or an increase of nitrite even when the reactor contained nitrifying microorganisms. The TAN and nitrite results in the RA unfiltered sample could be due to chloramine toxicity, as the ammoniated reactor did not experience such a condition in the operational period.

(a)
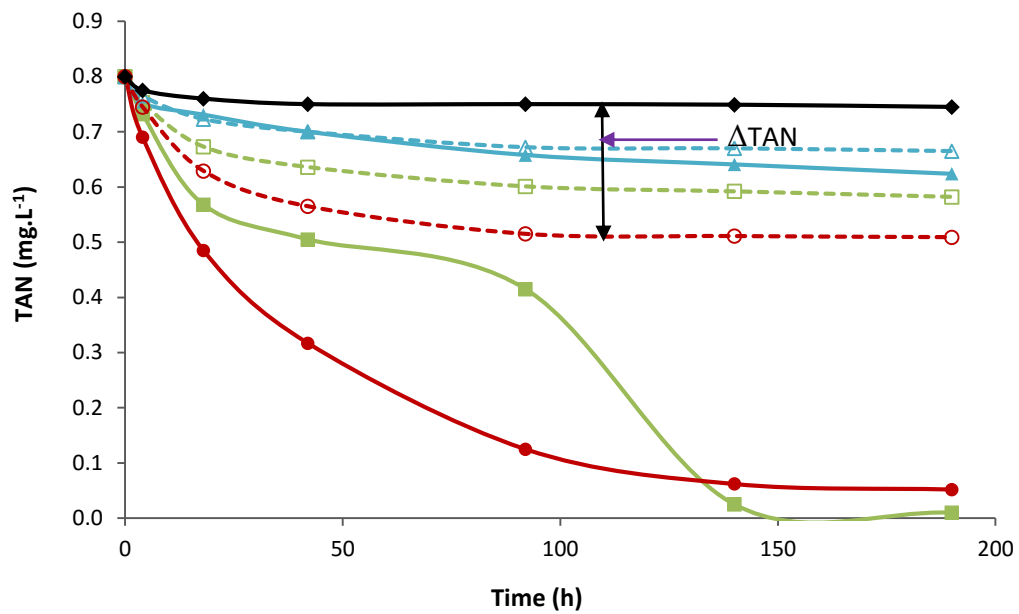
(b)
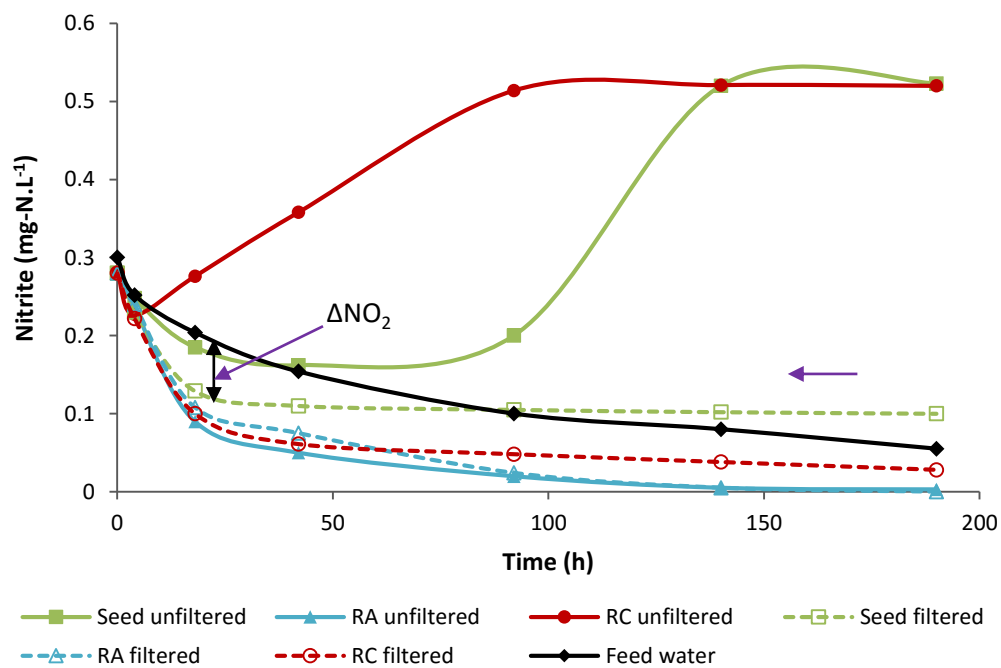
Figure 7.3: TAN and nitrite profiles of the ammoniated, seed and chloraminated reactor bulk water

Table 7. 2: The impact of CDP in filtered samples

Impact of SMP	Seed filtered	RC filtered	RA filtered
Δ NO$_2$-N (mg.L^{-1})	0.075	N/A	N/A
ΔTAN (mg.L^{-1})	0.157	0.238	0.079

The ΔTAN and ΔNO$_2$-N values calculated for the collected samples are recorded in Table 7.2. In the filtered samples, high ΔTAN values in the Seed filtered sample (0.157 mg.L^{-1}) and the RC filtered sample (0.238 mg.L^{-1}) were due to the CDP prompted auto-decomposition. The RA filtered sample recorded the lowest ΔTAN as there was no CDP impact. Similarly, ΔNO$_2$-N value in the Seed filtered sample (0.075 mg.L^{-1}) indicated CDP induced nitrite oxidation. However, due to the fast decay of nitrite in RC and RA filtered samples, ΔNO$_2$-N values cannot be calculated.

7.3.3 Bacterial community structure analysis

Diversity analysis

A total number of 32,23,047 sequences were generated from 3 different samples (ammoniated, chloraminated and seed water) and after removing the short and chimera sequences, 30,37,350 sequences (nearly 95% of the total sequences) were used to examine bacterial diversity. The rarefaction curves generated at 97% sequence similarity highlight that a considerable percentage of the bacterial communities in each of the samples have been captured (Fig 7.4). Therefore, the results could represent the majority of the bacterial diversity in the tested water samples.
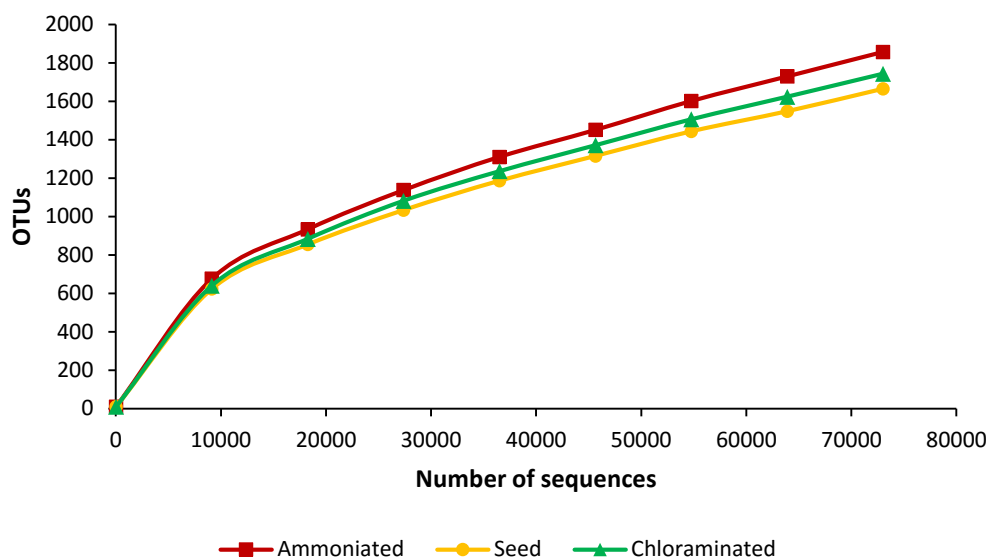
Figure 7.4: Rarefaction curves generated based on 97% sequences similarity for the chloraminated, ammoniated and seed water samples

The Shannon's diversity index indicates the species diversity and evenness while the Chao1 represents the richness of the species in the microbial community. According to the results presented in Table 7.3, bacterial species richness (Chao1), PD and bacterial diversity (Shannon index) were the highest in the ammoniated reactor. After introducing ammonia with low DOC containing water (i.e.; operating ammoniated reactor from the severely nitrified seed water), Shannon, Chao 1 and species diversity indices increased when compared with the seed water. However, chloraminated reactor increments in similar indices are lower than the ammoniated reactor. Additionally, species diversity reduced with the addition of chloramine (i.e. maintaining chloraminated reactor started with seed water) compared to the seed water sample. As chloraminated reactor maintained with the higher chloramine level than seed water, the growth of the bacteria may have suppressed in that reactor. Similar type of observation was made by Sathasivan, Fisher and Tam (2008) as they reported decrease of bulk water bacteria population with the increase of chlorine concentration.

Table 7.3: Bacterial community richness and diversity indices for ammoniated, chloraminated and seed water samples

Sample	PD* (Average)	Shannon Index*	Chao1* Average
Ammoniated	110.57	6.96	4183.73
Seed	103.35	6.66	3474.48
Chloraminated	101.84	6.89	3841.27

* Sequence per sample normalized to 73018

Similarity analysis

Figure 7.5a demonstrates the microbial community shifts during DOC reduction and chloramine (TCl and TAN) or TAN introduction in the chloraminated and ammoniated reactors, respectively, compared to the severely nitrified reactor. Only the severely nitrified reactor bacterial community shows the good relation with total chlorine, TAN and DOC. Clustering of bacteria is independent of the presence of nitrite.

(a)   (b)
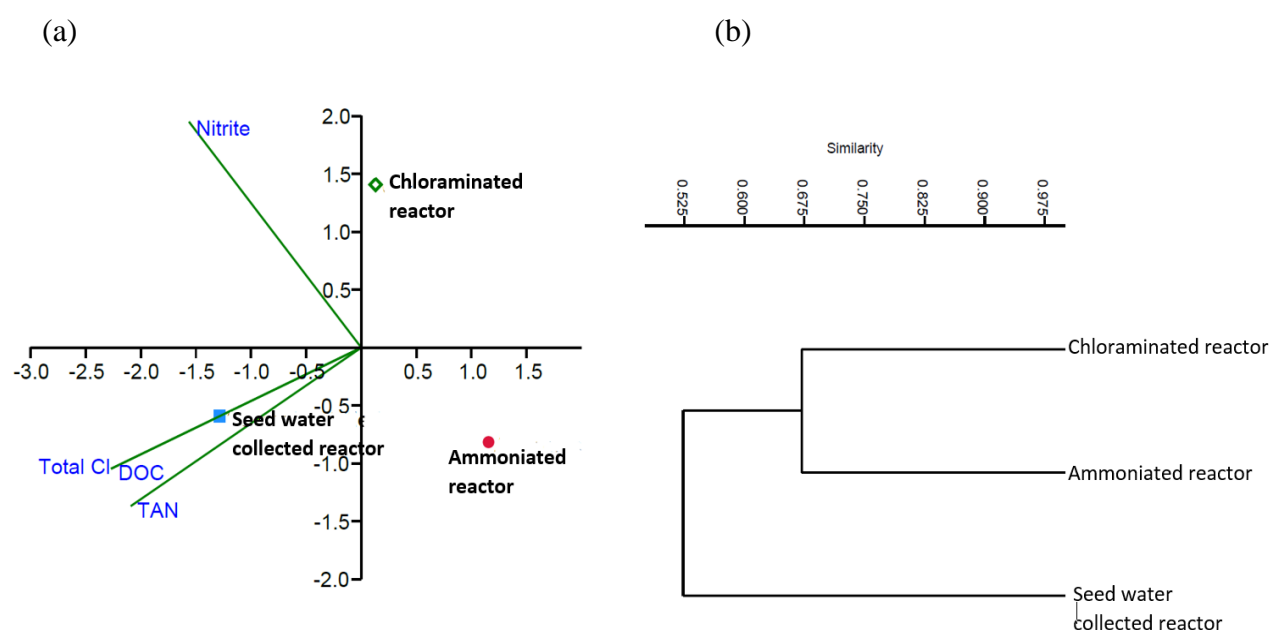
Figure 7.5: Similarity analysis (at 80% sequence similarity) of nitrified water samples collected from ammoniated, chloraminated and severely nitrified reactors based on Bray-Curtis similarity index (a) Canonical correspondence analysis (CCA), (b) Cluster analysis

According to Figure 7.5b, chloraminated and ammoniated reactors show high bacterial community similarity (67%). Even though all three water samples were in severely nitrified conditions, severely nitrified reactor shows around 52% bacterial community similarity with chloraminated and ammoniated reactors. This could be due to the DOC differences between the nitrified reactors. Advance analysis such as sequencing at 97% similarity would be more meaningful and could detect further similarity and variability among the reactors.

Bacterial community classification

The bacterial diversity of the collected samples was spread across a total 24 bacterial phyla. However, the highest number of sequences belonged to phyla Proteobacteria followed by phyla Chlamydiae (6 to 12%), Bacteroidetes (1.5 to 11%), Nitrospirae (0.5 to 11%), Cyanobacteria (1.5 to 2%), Planctomycetes (1 to 2%) and Actinobacteria (0.5 to 1.5%).

In both chloraminated and seed reactors, more than 80% (Fig. 7.6) of the bacterial population belongs to phyla Proteobacteria. The Proteobacteria population is less than 65% within the ammoniated reactor. The Bacteroidetes population was significantly high in the ammoniated reactor sample (11%) compared to the seed (0.6%) and chloraminated (1%) reactors samples. A similar result was recorded for Nitrospirae, they recorded 10, 0.2 and 0.3% for ammoniated, chloraminated and seed samples, respectively. All other phyla were recorded in minor values and no major differences were observed between all reactors.
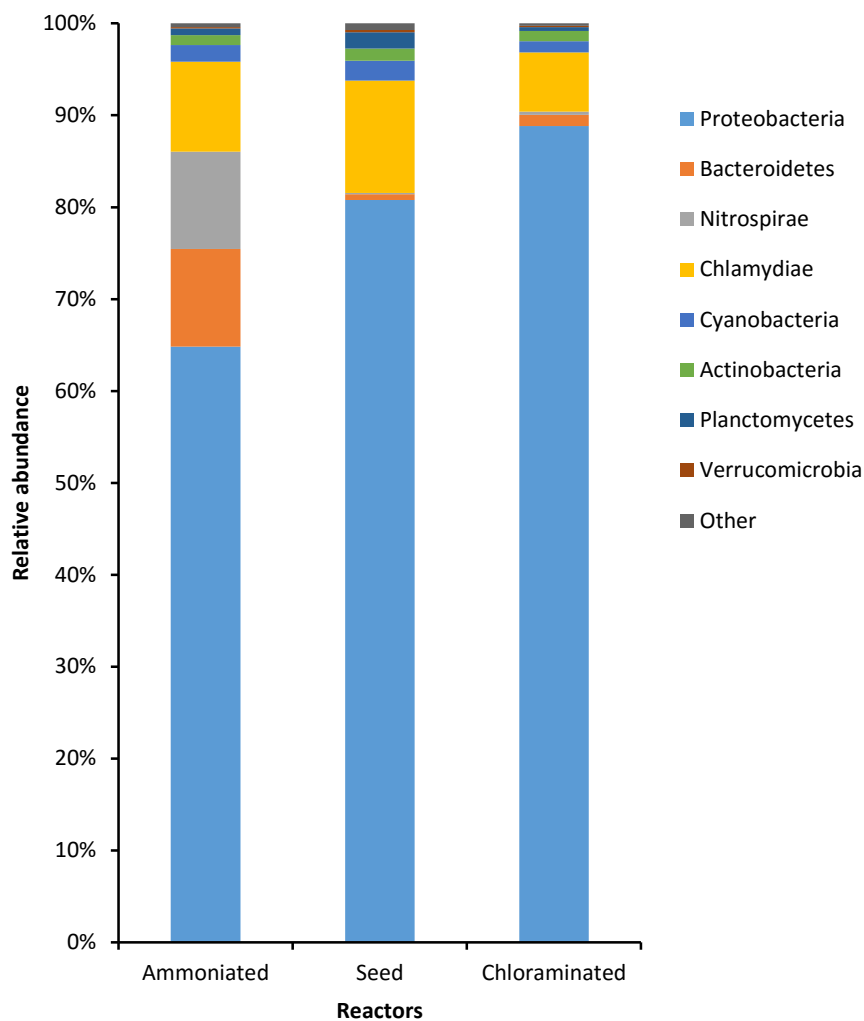
Figure 7.6: Relative abundance of bacterial phyla in three reactors

The relative abundance of the bacterial community in the ammoniated reactor, in the chloraminated reactor and in the seed water collected reactor based on their class level is presented in Fig. 7.7. In all three reactors, the dominant bacterial class is Alphaproteobacteria, which includes heterotrophic bacteria. Seed water recorded the highest relative abundance (67%) of Alphaproteobacteria whereas their abundances were 50% and 34% in the chloraminated and ammoniated reactors, respectively. The second largest class in chloraminated and ammoniated reactor is Betaproteobacteria whereas Clamydiia (human and animal pathogens (Molmeret et al. 2005; Thomas et al. 2008)) is the second largest class in the seed water collected reactor. AOB, such as *Nitrosomonas spp* and *Nitrosospira spp* belong to class Betaproteobacteria. It is also one of the leading bacterial classes in the seed water collected reactor as well (the third largest group in the nitrified reactor). In the ammoniated

reactor, Nitrospira (NOB) and Saprospirae (fermenting bacteria) were significantly high when compared to the chloraminated reactor and the seed water collected reactor. Gammaproteobacteria which includes photoautotrophic bacteria (e.g., purple sulfur bacteria) and heterotrophic bacteria (e.g., *Ecoli*, *Pseudomonas)* abundance is high in the ammoniated and chloraminated reactor (8%) when the seed water collected reactor recorded only 2%. All other classes were presented in minor proportions and are belong to classes such as Chloroplast (photosynthetic bacteria) and Actinobacteria (terrestrial or aquatic bacteria) and Planctomycetia. However, major differences among reactors cannot be seen.
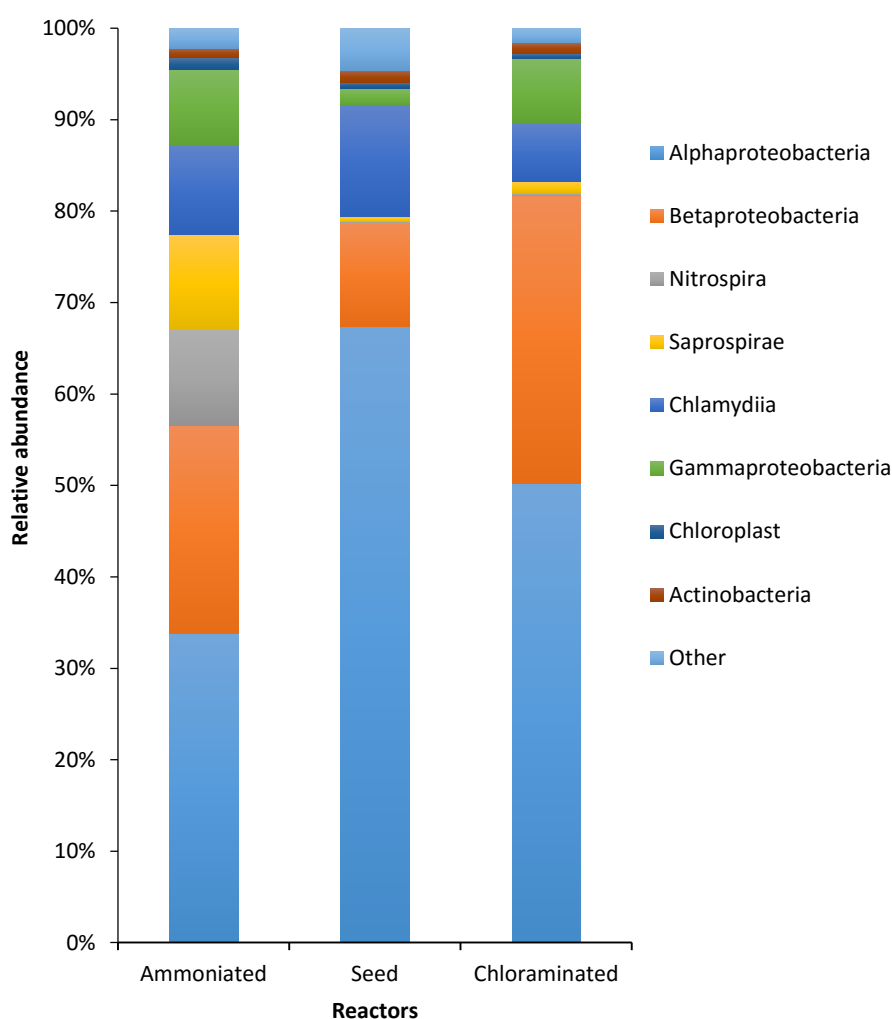
Figure 7.7: Relative abundance of bacterial classes in three reactors

Even with the low DOC level (~ 0.1 mg-C.L^{-1}) in the ammoniated reactor and the chloraminated reactor, heterotrophs are high. Assimilable organic carbon (AOC) is one of the most significant aspects affecting the re-growth of microorganisms in drinking water (Liu, X

et al. 2015). Therefore, AOC level should be high in all reactors as heterotrophic bacteria can be seen as the largest class within all reactors. As ammoniated and chloraminated reactors were nourished with the feed water prepared with nutrients added milli-Q water, the only way of having abundant AOC is from bacteria grown in the reactors (Fig.7.8).
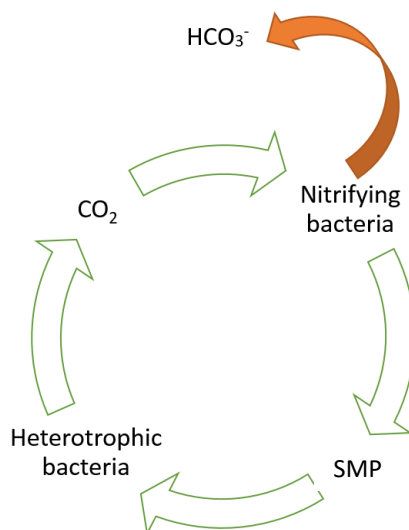
Figure 7.8: Relationship between heterotrophic and nitrifying bacteria for the survival

EPS which are produced by biofilm consist of humic and fulvic acids, polysaccharides, proteins, exocellular enzymes, DNA, etc. Similarly, (Herath, Torres & Sathasivan 2018)confirmed the presence of chloramine decaying proteins (CDP) when severe nitrification occurs. When there is no external organic carbon available, SMP produced by nitrifiers provided the sole organic substrates for heterotrophic bacteria (Kindaichi, Ito & Okabe 2004; Okabe, Kindaichi & Ito 2005). Therefore, one of the major tasks of these SMP is to provide the nutrients for surrounding bacteria. Other than that, it has been a known fact that dead cells also provide DOC for bacterial growth (Duursma 1963; Lee, DY & Rhee 1997; Otsuki & Hanya 1972). Therefore, it can be concluded that the nitrifying bacteria, possibly secretes CDP and dead cells facilitate the growth of heterotrophic bacteria by providing the substrate, resulting in a high level of heterotrophic bacterial community in the reactors.
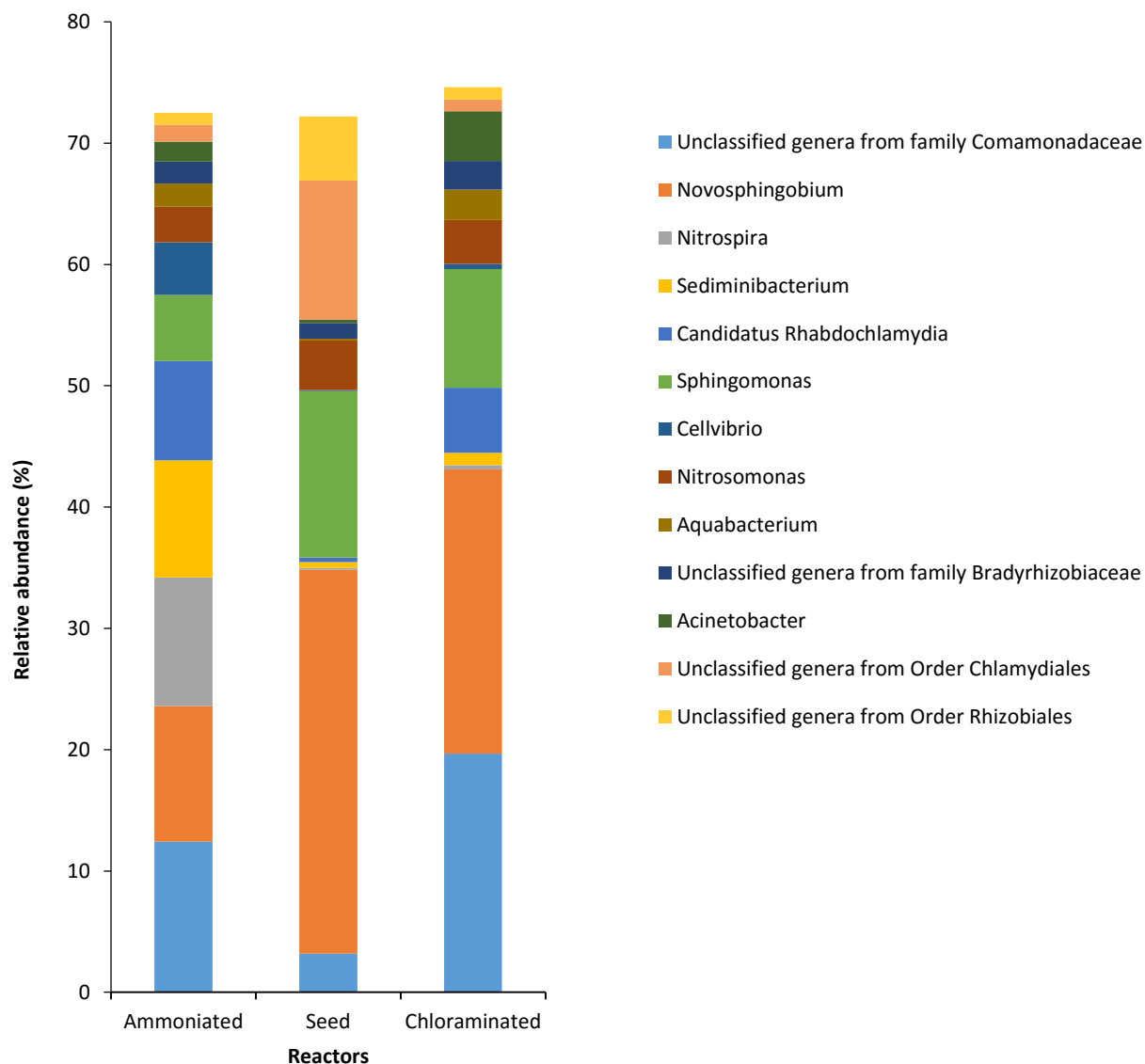
Figure 7.9: Relative abundance of bacterial genera in three reactors

There were over 394 bacterial genera identified in the chloraminated, ammoniated and seed water collected reactors. However, the most abundant bacterial genera belongs to Alphaproteobacteria (Novosphingobium*, Sphingomonas,* unclassified genera from Order Rhisobiales*, Hyphomycrobium, Phenylobacterium, Afipia*), Betaproteobacteria (*Nitrosomonas, Aquabacterium,* unclassified genera from Family comamonadaceae), Gammaproteobacteria (*Acinetobactor* and *Cellvibrio*), Nitrospira (*Nitrospira*), Saprospirae (*Sediminibacterium*) and Chlamydiia (*Candidatus Rhabdochlamydia* and unclassified genera from Order Chlamydiales) as shown in Figure 7.9.

In chloraminated and seed water collected reactors, the most abundant bacterial genusa was *Novosphingobium* (23% and 32%, respectively). *Novosphingobium* is a heterotrophic bateria

and was detected in chlorinated drinking water systems as one of the abundunt genera (Gomez-Alvarez et al. 2016). The abundant level in ammoniated reactor was low as 11% compared to the other two reactors. Therefore, the chloramine residual could affected the *Novosphingobium* community abundance among the reactors.

Gram-negetive, chemoheterotrophic *Sphingomonas* also servived in all three reactors. However, the highest relative abundance was detected in the seed water (15%) when they were 10% and 5% in the chloraminated and ammoniated reactors, respectively. Several authors (Krishna, Sathasivan & Ginige 2013; Noguera et al. 2009; Regan, John M et al. 2003; Regan, John M, Harrington & Noguera 2002; Williams et al. 2004) reported the dominancy of *Sphingomonas* in chloraminated systems. Furthermore, Liu, R et al. (2012) reported the production of exopolysaccharides by family Sphingomonadaceae, which includes the genera *Sphingomonas* and *Novosphingobium*.

An unclassified bacteria from family Comamonadaceae were detected with relative abundances of 11% and 23% in ammoniated and chloraminated reactors, respectively . However, in the seed water, it was <3%. Members of the Comamonadaceae group are often found in different environments including drinking water, lake water, activated sludge and soil (Lautenschlager et al. 2013). Some species (e.g. *Hydrogenophaga, Acidovorax*) from Family Comamonadaceae was reported as one of the abundant betaproteobacterial groups in the drinking water systems (Pinto et al. 2014). According to the observed results, their abundance was high when the DOC level is low. However, presence of chloramine did not affect the existance of family Comamonadaceae. Similarly, *Aquabacterium* belongs to the same family presented only in chloraminated and ammoniated reactor. *Aquabacterium* mainly presents in drinking water systems and forms EPS even under oligotrophic conditions (Manz, Kalmbach & Szewzyk 2015). Other bacterial types which only detected in chloraminated and ammoniated reactors are *Acinetobacter* and *Candidatus rhabdochlamydia.*

Nitrispira was presented in ammoniated reactor, but not in seed water collected and chloraminated reactors. This bacteria has the ability to perform complete oxidation of ammonia to nitrate (i.e., it is a complete ammonia oxidizer-comammox) in drinking water systems (Daims et al. 2015; Pinto et al. 2016; van Kessel et al. 2015; Wang, Y et al. 2017). Based on the chemical characteristics observed (Table 7.1), only ammoniated reactor demonstrated the complete nitrification condition and therefore, the results are consistant. Moreover, no chloramine condition in ammoniated reactor could facilitated the growth of Nitrospira. Apart

from that, *Nitrosomonas* was detected in similar abundance in all reactors. Furthermore, nitrifiers produce EPS or/and SMP (Kindaichi, Ito & Okabe 2004; Ohashi et al. 1995; Rittmann, Bruce E, Regan & Stahl 1994) which are primarily consisting of polysaccharides, proteins, nucleic acids and lipids (Flemming & Wingender 2010; Sheng, Yu & Li 2010) in certain scenarios.

In addition, uncharacterised bacteria from family Bradyrhizobiaceae and order Rhizobiales presented in all reactors. The highest relative abundance (7%) presented in the chloraminated reactor while seed and ammoniated reactors detected with ≈3%. *Bradyrhizobium* strains and Rhizobiales produce EPS while degrading organophosphorus pesticides (Abd-Alla 1994) and could be relevant to the CDP.

*Sediminibacterium (*10%*)* and *Cellvibrio (*4%*)* were only detected in the ammoniated reactor. In most of the drinking water biofilm studies (Schwering et al. 2013; Wang, Haibo et al. 2012; Wu et al. 2015), *Sediminibacterium* is a key contributor on forming biofilms. In some chlorinated distribution systems *Cellvibrio* is one of the major bacterial type recognized (Liu, J et al. 2017). Ayarza, Mazzella and Erijman (2015) reported *Sediminibacterium* sp. growing under planktonic conditions expresses stress-related proteins due to the planktonic environment rather than growing as in biofilm conditions. However, their absence in the chloraminated reactor and seed water is not known.
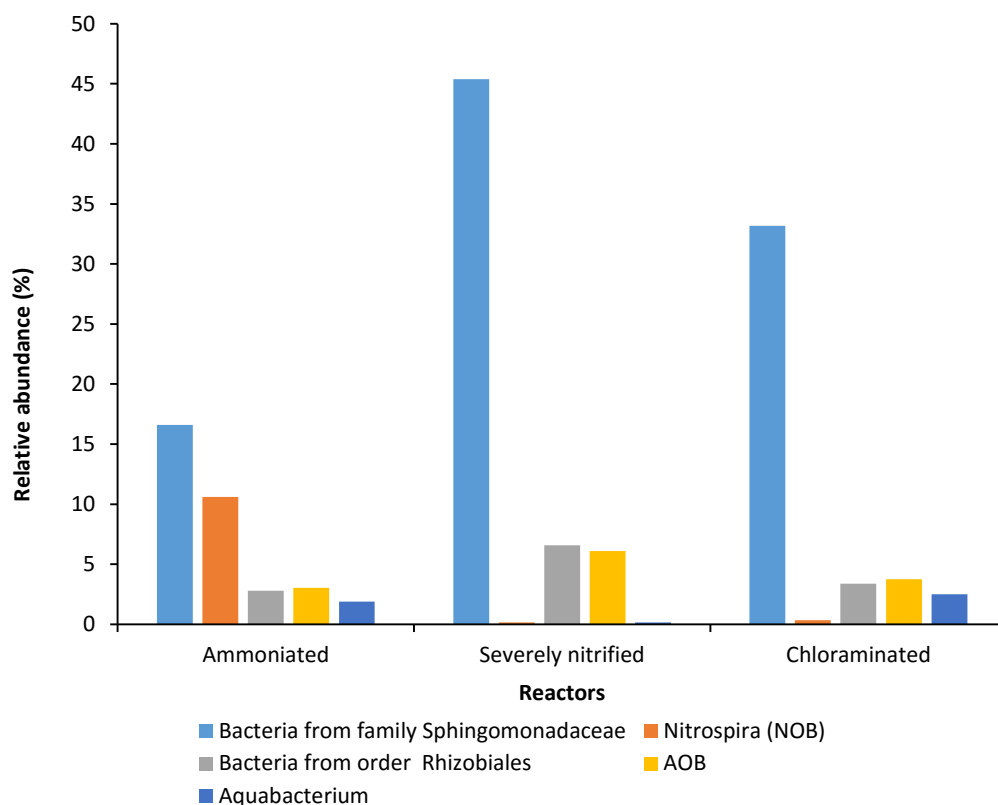
Figure 7.10: EPS producing bacteria identified in the three reactors

In summary, providing favourable conditions such as low DOC, chloramine stress for the production of CDP demonstrated significant difference in the decay profiles (total chlorine, TAN, nitrite) related to the presence of CDP. However, when comparing bacterial diversity and bacterial classification results, substantial difference cannot be identified in the reactor consisted with CDP (chloraminated reactor) and the reactor with no CDP (ammoniated reactor). Due to the existence of high chloramine level (0.45 mg.L^{-1}) in the chloraminated reactor, the relative abundancy of some bacterial species such as *Sediminibacterium*, *Candidatus Rhabdochlamydia* was supressed, compared to the no DCP reactor (ammoniated).

Even though both reactors demonstrated similar type of bacterial species, CDP can be produced in the chloraminated reactor by the same bacterial types presented in ammoniated reactor due to the chloramine stress. This argument is further supported as some of the bacterial types identified in this study which produce EPS (Fig. 7.10). The CDP can also be one of the components. Furthermore, due to the absence of disinfectant (chloramine) in ammoniated reactor, the number of cells within the tested sample could be higher than the other two reactor samples having disinfectant. The results presented are only based on the relative abundance and cannot indicate the actual number of bacterial cells present in each sample. Therefore, the

tested samples treated with chloramine may consist lower level of total bacterial cells. Moreover, it is a known fact some of the bacterial species significantly enhanced the EPS production under stress conditions (Liu et al. 2004). Therefore, further studies in relation to CDP identification and the EPS producing bacteria (*Bradyrhizobium,* family Sphingomonadaceae, nitrifiers, *Aquabacterium*) are essential.

7.4 Conclusion

Decay characteristics and bacterial communities in ammoniated, chloraminated and seed water samples were studied. Chloramine decay profiles with TAN and nitrite loss profiles confirmed the presence/activity of CDP only in the chloraminated reactor and seed water samples. Major conclusions made from the bacterial community analysis are:

- DOC level does not interfere the heterotroph bacterial community, as Alphaproteobacteria (heterotrophs) is the dominant bacterial class in all three reactors.

- Nitrifiers could provide the substrate for the heterotrophic bacterial growth, by releasing/producing SMP.

- A major variance in bacterial diversity and species cannot be seen in reactors maintained with chloramine (chloraminated reactor) and ammonia (ammoniated reactor).

- Several EPS producing bacterial strains such as *Bradyrhizobium, Rhizobiales,* family Sphingomonadaceae, nitrifiers and *Aquabacterium* were detected in both the ammoniated and chloraminated reactors.

Chapter 8

Identification of proteins from chloramine decaying water -

A proteomic approach

8.1 Introduction

Disinfection plays a key role in ensuring the supply of high quality drinking water for customers, which is the foremost objective in the drinking water industry. Disinfectants inactivate pathogenic micro-organisms (primary disinfection) and suppress their regrowth in water distribution systems (secondary disinfection). Secondary disinfection needs long-lasting disinfectant residual. While chlorine is popularly used around the world as a primary and secondary disinfectant, the concern over toxic halogenated disinfection by-products (DBP) have made utilities look for alternative disinfectants such as monochloramine (popularly termed chloramine), which is the second most popular secondary disinfectant for its minimal formation of regulated DBP and the ability to last longer (Duirk et al. 2005; Vikesland, Ozekin & Valentine 2001).

Chloramine faces few challenges from chemical and microbiological agents. Auto-decomposition (very slow decay on its own) and reaction with any reducing agents decomposes chloramine, resulting in release of free ammonia leading to nitrification (Hoefel, Daniel et al.

2005; Kim, D-J, Lee & Keller 2006; Pressman et al. 2012; Regan, John M et al. 2003; Regan, John M, Harrington & Noguera 2002).

The nitrifying bacteria, the primary organisms responsible for nitrification are aerobic autotrophs and they oxidize nitrogen in to a two-step microbial reaction. Firstly, the ammonia is converted to nitrite (Eq. 8.1) by ammonia-oxidizing bacteria (AOB) like *Nitrosomonas. S*econdly nitrite is converted in to nitrate by nitrite (Eq. 8.2) oxidizing bacteria (NOB) such as *Nitrobactor* (Shammas 1986). Other than nitrifying bacteria, some of the archaea are also involved in the first equation step, oxidising ammonia in to nitrite (Hu et al. 2010; Schleper & Nicol 2010; Verhamme, Prosser & Nicol 2011). It is widely believed that nitrification is the main cause for the loss of chloramine in the distribution systems due to the formation of nitrite. Recently we have shown that other microbes may also decay chloramine (Herath, Sathasivan & Lam 2015).

$$1/6\ NH_4^+ + 1/4O_2 \longrightarrow 1/6NO_2^- + 1/3H^+ + 1/6\ H_2O^+ \qquad\qquad (Eq.\ 8.1)$$

$$1/2\ NO_2^- + 1/4O_2 \longrightarrow 1/6NO_3^- + 3H^+ \qquad\qquad (Eq.\ 8.2)$$
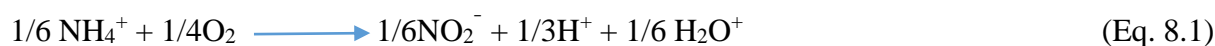
However, bacteria-free severely nitrified water samples from chloraminated drinking water distribution systems and the pilot scale systems tested by Krishna, Sathasivan and Sarker (2012) demonstrated accelerated chloramine decay. They highlighted evidence that chloramine decay could be accelerated mainly due to the presence of soluble microbial products (SMP) and could be the major factor of chloramine decay.

Previous studies clearly demonstrated that extracellular polymeric substances (EPS) can decay disinfection agents such as chlorine or chloramine (Wonoputri et al. 2015; Xue et al. 2013). Soluble Microbial Products are considered as soluble EPS and they are closely related with EPS that is bound to bacterial cells (Janga et al. 2007). As per Neu, Flemming and Wingender (1999) EPS is generally considered to be composed of proteins, carbohydrates, nucleic acids, lipids and humic substances. However, proteins and carbohydrates are the dominant components of EPS (Liu, Yan & Fang 2003; Neu, Flemming & Wingender 1999). A biofilm can be defined as a community of microorganisms adhering to a surface and surrounded by a complex matrix of EPS (Bridier et al. 2011; Laspidou & Rittmann 2002). Therefore, soluble chloramine decaying agents could also be detected in the biofilm samples.

Apart from that, there is enough evidence AOB are able to grow on residual and released ammonia in the presence of chloramines and produce nitrite and soluble organic compounds

(Furumai & Rittmann 1992; Liu, Yiwen et al. 2016; Pan & Umbreit 1972; Rittmann, Bruce E, Regan & Stahl 1994). Herath, Torres and Sathasivan (2018) proved that the chloramine decaying SMP or soluble chloramine decaying agents are proteins (30kDa<MW<50 kDa) and thus called them chloramine decaying proteins (CDP). They also reported that the impact of CDP generally decreased when the dissolved organic carbon (DOC) level of the feed water is increased.

The previous work I had conducted (Herath, Sathasivan & Lam 2015) with nitrified bulk water samples enabled the identification of several proteins (more than 25 proteins). Due to the identification of several proteins, the protein/s specifically related to accelerated chloramine decay could not be pin-pointed. Furthermore, it proved difficult for dedicated proteins (CDP) to be captured/separated for the identification process due to their low concentrations. Therefore, achieving high concentration for proteins in focus and elimination of unnecessary proteins while capturing CDP were the crucial matters that needed resolution. Furthermore, low DOC nitrified water resulted in more CDP impact (Chapter 6). Hence; in this current study, bulk water samples collected from the ammoniated and chloraminated reactors maintained with nutrient rich milli-Q water were used to extract CDP. Identical proteins observed in both reactors can be rejected, as they cannot be included as CDP. As bulk water protein concentration was too low for identification, a second experiment was conducted, where protein extraction was directed with biofilm samples. The extracted proteins were separated and identified using gel electrophoresis followed by Mass Spectrometry analysis.

8.2 Materials and methods

8.2.1 Sample preparation

Bulk water samples:

With the purpose of identifying the proteins from bulk water, 4 L from each reactor (chloraminated and ammoniated) were concentrated by filtering through 50 kDa ultrafiltration discs (Biomax® Polyethersulfone membrane, 63.5 mm, PBQK06210, Millipore, USA) followed by 30 kDa ultrafiltration discs (Biomax® Polyethersulfone membrane, 63.5 mm, PBQK06210, Millipore, USA) using the Stirred Ultrafiltration Cell (Millipore, 8200, CAT no 5123, USA). After ultrafiltration, 5 ml of concentrated sample (retantate sample) was collected for protein identification. The 30 kDa ultrafiltration discs used to prepare concentrated samples

were washed 5-6 times with 1 mL of milli-Q water to extract the proteins possibly bound to the 30 kDa ultrafiltration discs (filter paper washed sample).

To remove any suspended particles from the prepared water samples, samples were centrifuged at 5000 g using table top centrifuge (Allegra 12 centrifuger, Beckman coulter) for 5 min. Then all samples were frozen at -80 °C before subjecting to freeze drying process- lyophilisation. Lyophilisation was performed on CHRIST freeze dryer (Burkert, Germany) at -42 °C at 10 mbar for overnight. Resulted dried material from lyophilisation was resuspended in minimal volume (50 μl) of distilled water for protein concentration determination.

Biofilm samples:

The bulk water from the Chloraminated and ammoniated reactors that were tested in this experiment provided less protein concentrations/spots for the CDP identification process. Therefore, the biofilm was extracted from the reactor wall and used to conduct protein identification tests. In order to examine the protein profiles from biofilms, samples were collected aseptically from the reactor walls using cell scrapers (28 cm length, Greiner Bio-One GmbH, 541070, Germany) and stored at -30 °C until further experiments were carried out.

After the samples were prepared, protein assay was performed on above reconstituted bulk water samples and biofilm samples using fluorescent assay method EZQ (Invitrogen) to determine their protein concentration.
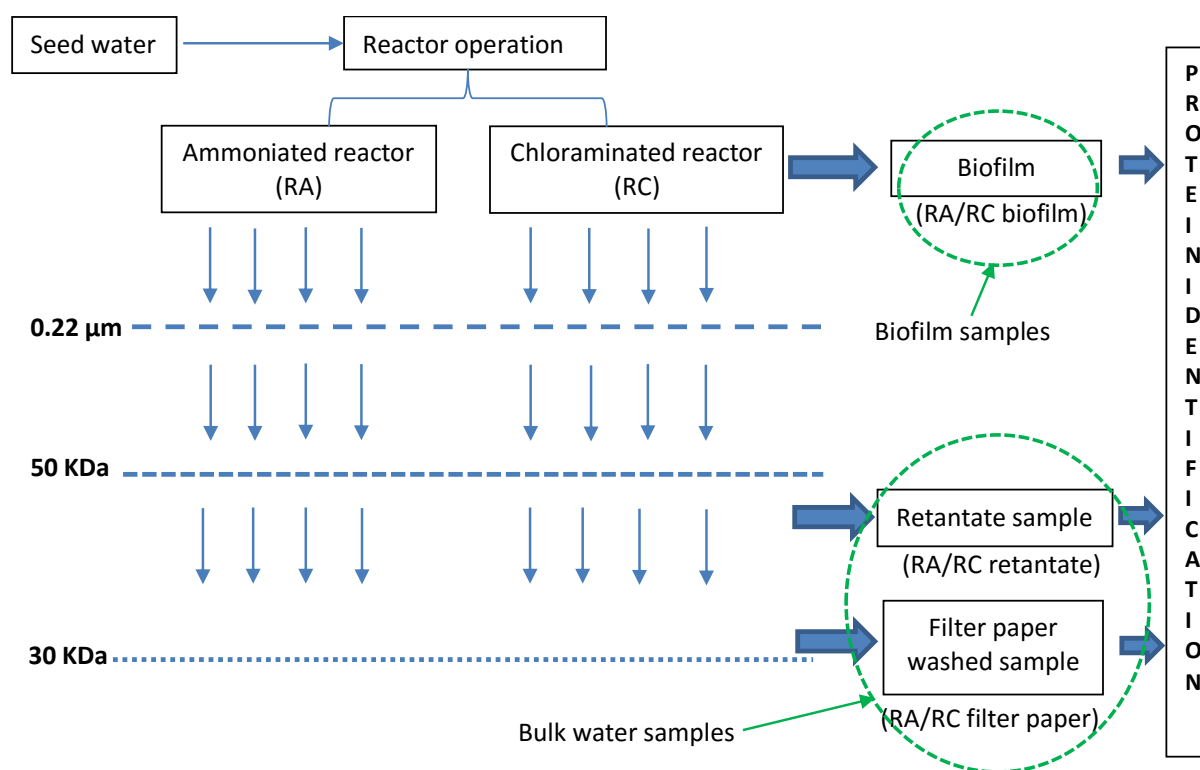
Figure 8.1: Schematic diagram showing the experimental approach to capture the active molecules from ammonia and chloramine treated water samples

8.2.2 Protein estimation using fluorescent dye method-(EZ-Q) assay

High sensitive fluorescent protein detection method (EZQ- Invitrogen Catalogue R33200) was used to determine the protein concentrations of the above prepared samples. Standard protein bovine serum albumin (BSA) stock (1mg/ml) was prepared in 2% sodium dodecyl sulphate (SDS) buffer to generate 12.5, 25, 50, 100, 150, 200, 250 and 300 µg proteins and used to generate protein standard graph. 2 µL of each above diluted proteins ware spotted in triplicates on a Whitman filter paper and immediately air dried to minimise sample diffusion. Similarly, 2 µL of 5 times diluted water protein in triplicates were spotted as unknown samples. After protein spotting, filter paper was transferred to a plastic container and fixed with 50 ml of 100% methanol for 5 min and left in a fume hood to dry the paper. EZQ protein quantitation reagent (50 ml) was added to the container and incubated for 30 min on a shaker (50 rpm) under dark conditions. Immediately after incubation, excess amount of dye from the filter paper was removed by washing the blot with a solution containing a mixture of 10% methanol and 7%

acetic acid solution three times, with an interval of 20 s between each wash. The resulted filter paper was scanned on '2 sec' settings using FUJI LAS-4000 scanner on excitation and emission settings of ~485/590 nm. Then the protein spot intensities were measured using Multi Gauge software (FUJIFILM Corporation, Tokyo Japan) to prepare a standard curve (See table 8.2) for determining the protein concentration. After protein estimation, samples were analysed on two-dimensional gel electrophoresis (2DE) (for bulk water samples) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (for biofilm samples).

8.2.3 Protein separation on bulk water samples

Two-dimensional gel Electrophoresis (2DE)

Two-dimensional gel electrophoresis is one of the most widely used tools for separating proteins based on their size and charge. As it is very useful to separate two proteins with identical molecular weights but different charges, bulk water proteins were separated by 2DE for identification process.

First dimension: Isoelectric focusing (IEF)

According to the protein concentration resulted, buffer containing 8 M urea, 2 M thiourea and 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) was added in to each protein sample. Next, a buffer which contains 8 M urea, 2 M thiourea, and 4% CHAPS along with 2% ampholytes was added to make the 1% final ampholytes concentration. For disulfide reduction, 2.6 mM tributyl phosphine/dithiothreitol (TPB/DTT) disulfide reduction buffer was added. Alkylation was conducted with acrylamide monomer stock conc. 5.6 M and working conc. 230 mM. Consecutively, this was incubated for 1 h at 25 C.

For immobilised pH gradient (IPG) rehydration, treated protein was dispensed into the rehydration tray and peeled non-linear, 7 cm IPG strips (Bio-Rad, ReadyStrip IPG 7 cm, pH 3-10NL, Cat no 163-2002) were placed onto the sample with the gel side down, without creating any air bubbles. To prevent the protein evaporation, 2-3 mL of mineral oil (Cat no:1632129, Bio-Rad) was overlayed each of the IPG strip. The rehydration tray (from Bio-rad) was covered with a lid and incubated overnight (no longer than 16 hrs) on a level bench at room temperature for loading the protein samples and rehydrating the gel. The final rehydration volume after

disulphide reduction and alkylation treatment was maintained to 125 µL for 7 cm IPG strips to safeguard the condition that gels used for 2DE were not overloaded.

Paper wicks (cut from Whitman paper) were placed on a dry PROTEAN® IEF focusing tray using forceps at each end of the channels so that the wire electrode is covered. Wicks were wetted with 10 µl deionised water. After removing the cover of the IPG strips containing equilibrium tray, the strips were held vertically for about 7-8 s and mineral oil was removed by gentle blotting of the IPG strip tip. Then the IPG strips were transferred with correct polarity in to the corresponding channels in the focusing tray maintaining gel side down and covered with 2-3 mL of fresh mineral oil. Finally, focusing try was placed in to the IEF cell (Bio-Rad Protean IEF cell), lid was closed and IEF was carried out with appropriate protocol at 20 ˚C.

A constant current of 50 µA/gel was applied with desalting (250 V for 15 min) and linear ramping (4000 V over 2 h) with change of electrode wicks each 30 min during linear ramping to ensure trace salt removal and smooth ramping. Then a constant voltage of 4000 V was applied for 37500 volt-hours.

Prior to the second dimension, it is necessary to equilibrate the IPG strips in SDS containing buffers. Therefore; the IPG strips were removed from the mineral oil by placing them (gel side up) onto a dry filter paper and blotted with a second piece of wet filter paper. Firstly, 2.5 ml of pre prepared IPG equilibration buffer 1 (6 M urea, 20% glycerol, 0.375 M Tris-HCl, 2% SDS) accompanied with 2% DTT was added on to an equilibration/rehydration tray, using one channel per IPG strip. Then the blotted IPG strips (gel side up) were transferred in to the equilibration/rehydration tray and shaken (on a slow shaker mode) for 10 min. The used equilibration buffer 1 was discarded completely and equilibration buffer 11 (6 M urea, 20% glycerol, 0.375 M Tris-HCl, 2% SDS with 2.5% iodoacetamide) was added to each IPG strip and shaken for 10 min as previously. After incubation, the used buffer was discarded and the strips were ready for the second dimension.

Second dimension: SDS- PAGE

It is necessary to prepare the polyacrylamide gel before the rehydration process as it has to be left overnight for polymerisation. Therefore, 12.5 % polyacrylamide gel was cast as a separating gel (sometimes called resolving or running gel), topped by a stacking gel and secured in an electrophoresis apparatus.

Polyacrylamide gel preparation

In the preparation of separating gel process, gels were buffered with 1.5 M Tris buffer (pH 8.8), 10% (w/v) SDS and were polymerised with 10% (w/v) ammonium persulphate (APS) and 100% (v/v) tetramethylethylenediamine (TEMED). Catalyst 0.1% (w/v) APS and 0.1% (v/v) TEMED was used in the polymerisation process. Using two clean glass plates and two 0.75-mm spacers, the glass-plate sandwich of the electrophoresis apparatus was assembled according to manufacturer's instructions, before locking the sandwich to the casting stand. To prevent the polymerisation of the separating gel in the prepared flask, separating gel solution was immediately applied to the sandwich along an edge of one of the spacers using a Pasteur pipette until the height of the solution between the glass plates as ∼11 cm. Then the top of the gel was covered with the H_2O-saturated isobutyl alcohol layer (∼1 cm thick) by gently layering the isobutyl alcohol against the edge of one and then the other of the spacers. Formation and removal of bubbles were achieved using thinly layered isobutyl alcohol which created and even gel surface to form and remove bubbles. Polymerisation of the gels was completed overnight at 4 °C. After the polymeration, the gels were gently washed with ultra-pure water using a wash bottle, to achieve a flat surface, stacking gel was overlayed until the height of the solution in the sandwich was ∼1 cm from the top of the plates. The stacking gel was prepared using 5% (w/v) acrylamide (1:37.5 crosslinker: monomer) / 375 mM Tris Buffer (pH 8.8) / 0.1% (w/v) SDS / 0.001% (w/v) bromophenol blue. The gel plates were placed for 30 to 45 min at room temperature, allowing the stacking gel solution to get polymerized. Excess water remaining inside the IPG well on the gel was removed using blotting paper. Then the gel cassettes were placed at an angle on the stand for ideal IPG strip loading.

SDS-PAGE

IPG strips were placed on top of SDS-PAGE gels (12.5% T, 2.6% C acrylamide/bis-acrylamide resolving gel and 5% T, 2.6% C stacking gel). Both resolving and stacking gels that had been buffered using 375 mM tris-HCl (pH 8.8), comprised of 0.1% SDS. IPG strips were sealed to stacking gel with agarose solution (0.5% low-melting agarose, 375 mM tris-HCl (pH 8.8), 0.1% SDS) and then electrophoresis was implemented at 4 °C at 150 V for migration into stacking gel and later resolved at 90 V.

Gels were fixed with 10% methanol containing 7% acetic acid buffer for 1 hr with continuous shaking after the electrophoresis. After the gel was washed with distilled water for 3x20 min,

the gels were stained with a ready–to–use SYPRO Ruby IEF (Bio Rad) sensitive, fluorescent stain for 20 h. Sypro ruby protein stain is excited around its two excitation peaks at ~ 280 nm and ~ 450 nm and has an emission maxima at ~ 610 nm. The excessive stain was washed with a 0.5% NaCl solution, at 5x 15 min intervals and then the gels were ready for imaging. All imaging was done at 100 μm resolution using the scanner-based FLA-9000 imaging system (FUJIFILM Corporation). After washing with distilled water, gels were again stained with colloidal Coomassie Brilliant Blue (cCBB) and followed the same washing procedure before imaging on FLA.

Preparation of colloidal Coomassie Brilliant Blue (cCBB) dye solution:

Initially a stock solution of phosphoric acid (40%), ammonium sulphate (40%) and Coomassie Brilliant Blue (CBB) dye solution (2%) were prepared in distilled water and stored at room temperature. Fresh staining solution was prepared by mixing phosphoric acid (fc 2%), ammonium sulphate (fc 10%) dye solution (fc 0.1%) and methanol (100% v/v) in distilled water before use. 50 mL of this solution was used to stain a single gel. Gels were left along with the staining solution, on a shaker for 20 h. Next day, carefully removed staining solution from each gel were washed with distaining solution (0.5 M sodium chloride in distilled water) to remove excessive dye from the gels, for five times with an interval of 15 min between each wash.

8.2.4 Protein separation on biofilm samples

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

In the current study, complex mixture of proteins from the samples are separated to identify different proteins. Biofilm samples from chloraminated and ammoniated reactors were subjected to SDS-PAGE. Before subjected to SDS-PAGE analysis, concentrated water samples were defrosted and centrifuged at 3,000 rpm at 4 °C for 10 min (ROTINA 420R Ultracentrifuge) to form a pellet. Protein samples were resuspended in sample buffer containing 0.5 M Tris solution (pH 6.8) with 20% glycerol, 0.2 M DTT, 4% w/v SDS, 0.001% (w/v) bromophenol blue dye and denatured by boiling for 2 min at 100 ºC in microcentrifuge tubes.

Precast ready-made gels (mini PROTEIN TGX, Bio-Rad, P 10027885, T64134072, 8-16% 10 well comb, 25 µL/well) were used in SDS-PAGE. Therefore, before loading the samples, gels were rinsed with electrophoresis buffer (0.125 M Tris, 0.96 M glycine and 0.5% w/v SDS) in order to remove preservatives and fixed into gel cassettes (Mini-PROTEAN Empty Cassettes

from Bio-Rad). The resuspended protein samples were loaded into wells using gel loading tips (Eppendorf® Pipette Tips). Similarly, 25 µl of protein standards (Prestained SDS-PAGE Standards, broad range, CAT no:1610318, Bio-Rad) was also loaded. Equal volume of sample buffer was added to any empty wells to prevent spreading of samples to adjoining lanes. Then the sample loaded gel sandwich attached cassettes were inserted in to the vertical electrophoresis cell (mini- PROTEAN® tetra cell) following manufacturer's instructions and slowly filled with electrophoresis buffer.

Gels were run by applying 150 V for 2-3 min initially to allow protein to enter into ready-made gels followed by 90V for 3 h or until the tracking dye bromophenol blue front reached to the bottom of the gel. Gel cassettes with readymade gel plates were removed from the electrophoretic tank, then briefly rinsed with distilled water.

The gels were carefully removed from the plates and proteins were fixed with a buffer containing 10% (v/v) methanol and 7% (v/v) acetic acid for 1 h. After fixing, gels were washed three times with distilled water, 20 min between each wash, on a shaker at 50 rpm. After the third wash, gels were stained with cCBB staining solution for 20 h.

8.2.5 Protein identification on Mass spectrometry

Protein digestion and peptide extraction

Processing and digestion of gel spots and liquid chromatography-multistage mass spectrometry (LC-MS/MS) were carried out using Xevo QTOF (Waters). The protein spots excised from cCBB stained gels were destained by washing twice with 50% acetonitrile/50 mM NH_4HCO_3, pH 9 dehydrated with 100% acetonitrile and rehydrated in 20 µL of trypsin (12.5 ng.µL^{-1}). After overnight incubation with trypsin, the peptide solution was collected and 15 µL was transferred to an auto sampler vial for analysis. LC-MS/MS analysis was carried out.

MS/MS analysis

In brief, 3 µL of each peptide solution were loaded onto a Waters Xevo QToF mass spectrometer at 5 µL/min a Waters C18 Symmetry trapping column (180 µm x 20 mm) and desalted at this flow rate for 3 min using 1% acetonitrile in water (both with 0.1% formic acid modifier) as solvent. The peptides were washed off the trap at 400 nL/min on to a Waters C18 BEH analytical column (75 µm x 100 mm), packed with 1.7 µm particles of pore size 130 Å.

After separation, the peptides were analysed using tandem mass spectrometry, implementing an emitter tip that tapers to 10 µm at 2300 V. A Data Directed Acquisition (DDA) experiment was performed which continuously scanned for peptides of charge state 2^+ - 4^+ with an intensity of more than 50 counts, and a maximum of 3 ions in any given 3 s scan. Selected peptides were fragmented and the product ion fragment masses measured with a MS/MS mass range of 50-2000 Da.

Database search

The MS/MS data files were analysed with above mentioned settings using Mascot Daemon and checked against the SwissProt and Ludwig databases. The results of the search were filtered by including only protein hits with at least one unique peptide, and excluding peptide hits with a p-value greater than 0.05. Peptides identified by Mascot were further validated by manual inspection of the MS/MS spectra for the peptide to ensure the b- and y-ion series are sufficiently extensive for accurate identification.

8.3 Results

8.3.1 Protein estimation by fluorescent method

Protein concentrations in bulk water and biofilm samples were estimated using standard curve (Fig 8.2) are presented in Table 8.1
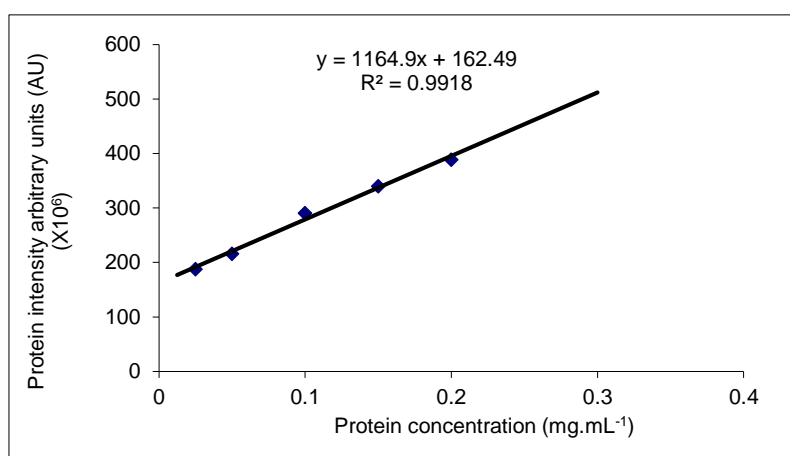
Figure 8.2: Standard curve prepared using BSA as a standard protein

It was observed that the amount of protein recovered from bulk water was very low (<0.07 mg.mL^{-1}) when compared with that from biofilm. Furthermore, proteins extracted from the ammoniated bulk water were lower in concentration than the chloraminated bulk water in this assay.

Table 8.1: Protein concentrations from various samples. Water samples were prepared as per Section 8.2.1

Samples		**Protein concentration (mg.mL^{-1})**
Bulk water	RC retantate	0.05
	RC filter paper	0.07
	RA retantate	< 0.02
	RA filter paper	<0.02
Biofilm	RC biofilm	3.17
	RA biofilm	1.19

8.3.2 Separation of proteins from bulk water

In order to visualise the protein differences between two sample extracts, 2DE was performed. Proteins were first focussed on a 3-10 border pH gradient followed by separation of proteins on 12.5% SDS-PAGE gels were shown in figures 8.3 (stained with Sypro ruby) and 8.4 (stained with cCCB). There was a significant protein spot difference between RC filter paper and RA filter paper samples as shown by red circles in Figure 8.3 (c).

(a)   (b)

(c)   (d)
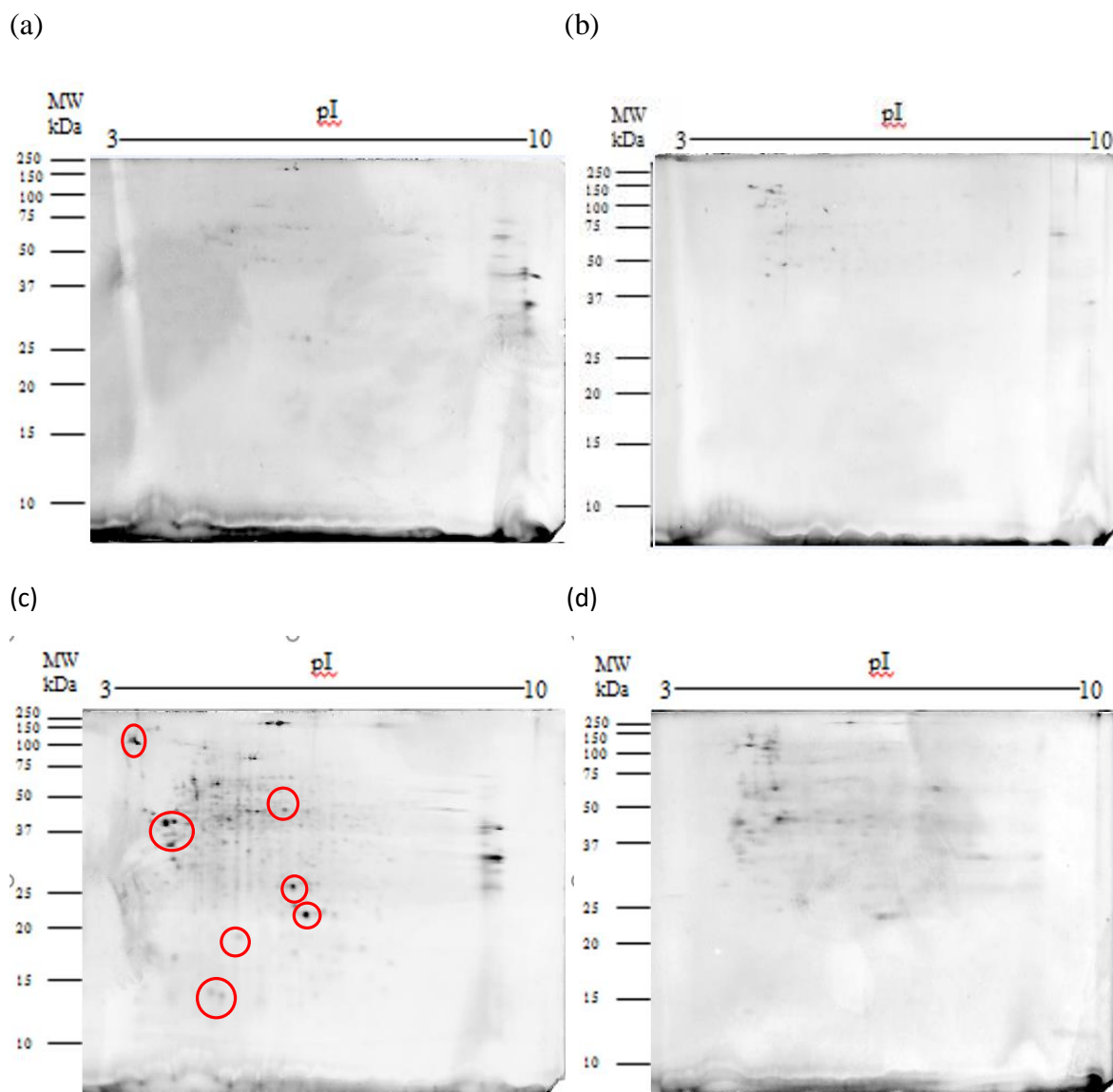
Figure 8.3: Analysis of proteins using two-dimensional gel electrophoresis stained with Sypro ruby. (a) RC retantate, (b) RA retantate, (c) RC filter paper and (d) RA filter paper. Red circles on RC gel represent the protein spots not detected on RA gel.

kDa = kilo Daltons, MW =molecular weight marker, pI   = pH 3 to 10 range.

Gels stained with Sypro ruby clearly showed the ammoniated samples do not show many protein spots on both retantate and filter paper extracted samples. On the other hand, the chloraminated proteins bound to filter paper showed a increase in protein spot numbers and the molecular weights of these proteins were in the range between 30 kDa and 75 kDa and pI range between 3.5 to 7.0 range.
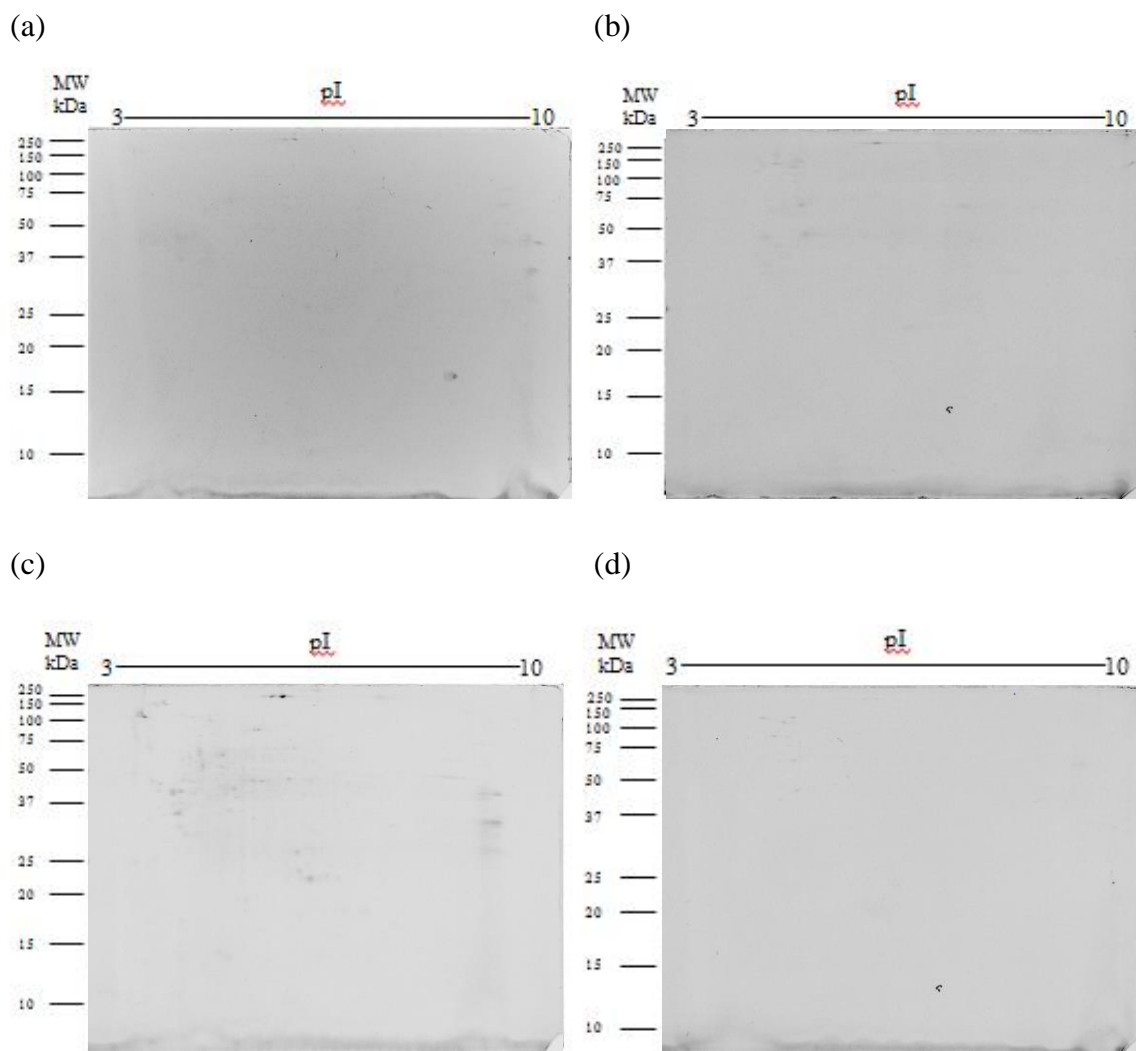
Figure 8.4: Analysis of proteins using two-dimensional gel electrophoresis stained with cCCB. (a) RC retantate, (b) RA retantate, (c) RC filter paper and (d) RA filter paper.

Similar to the gels results with Sypro ruby staining, cCBB stained gels noticeably showed the ammoniated samples do not show many protein spots on both retantate and filter paper extracted samples. However, the visible spot numbers on cCCB stained gels are lower than the Sypro ruby stained gels. On the other hand, the chloraminated proteins bound to filter paper showed increase in protein spot numbers; however, it is not significant as in Sypro ruby stained gels. The molecular weights of these proteins were also in the range between 30 kDa and 75 kDa and pI range between 3.5 to 7.0 range, which are similar to the gels stained with Sypro ruby.

8.3.3 Separation of proteins from bio-films

Proteins extracted from biofilms of chloraminated and ammoniated reactors showed clear separation of protein bands on precast 12.5% SDS-PAGE (Figure 8.4). The molecular weights of these proteins ranged from 20 to 250 kDa.

In chloraminated biofilm sample, 12 protein bands can be clearly visualised while only 9 protein bands from ammoniated bulk water sample (Fig. 8.5). Furthermore, the protein bands recognized from chloraminated biofilm sample are located in different molecular weight positions compared to the ammoniated biofilm sample. As an example; between 40-75 kDa, the protein bands resulted are C7, C8, C9, C10. However, the correspondent bands cannot be identified from the ammoniated biofilm sample. Bands on different molecular weight positions (e.g. A7, A8, A9) were identified from ammoniated biofilm sample. These protein bands from both waters were cut and identified using Mass spectrometry.
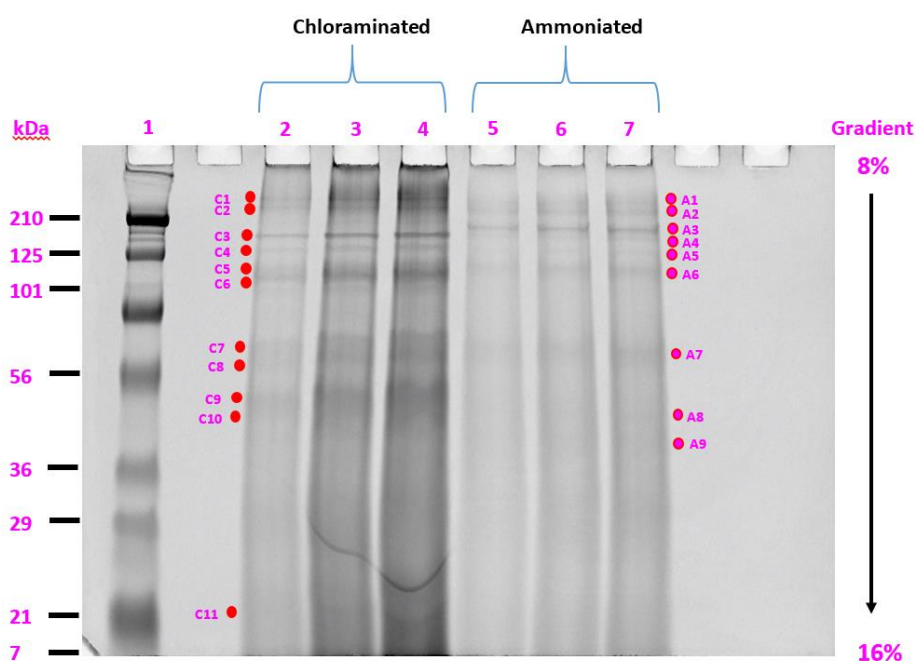
Figure 8.5: Protein from chloraminated and ammoniated proteins were separated on 12.5% pre-cast gels and stained with cCBB to visualise protein bands

Lane 1 represents the molecular weight markers. Lanes 2, 3 and 4 represent the RC samples obtained from biofilm loaded as 31.7 µg (10 µL), 47.6 µg (15 µL) and 63.4 µg (20 µL). Lanes 5, 6 and 7 represent the RA samples from biofilm loaded 11.9 µg (10 µL), 17.9 µg (15 µL) and 23.8 µg (20 µL).

8.3.4 MS analysis

Mass spectrometer analysis were not conducted with bulk water samples as the detected protein spots were not enough for the proper comparison to achieve the specific proteins related to accelerated chloramine decay. Protein samples prepared from biofilms were used to identify the protein bands using the mass spectrometer after separation on SDS-PAGE gels (Table 8.2). Protein spot numbers such as C1, C 2, C3…and A1, A2, A3… represent the nominated protein bands cut from SDS-PAGE gel as shown in Fig. 8.5.

According to data presented in Table 8.2, most of the proteins identified by MS and later matched with the SwissProt data base the and Ludwig database are from bacteria. Score value represents the confidence of the match. The higher the score number, the more the confidence of the identity of the protein.

Table 8.3 presents the summary of identified proteins and bacterial species from the chloraminated reactor water samples with high score values in interested size range (CDP size range of 30-50 kDa).

Table 8.2: Protein identification results obtained from MS

Condition	Protein pot no.	MASCOT ID	Name of the identified protein	Score	MW (kDa)	Calculated pI	% Sequence coverage	Matched sequence	Identified bacterial species
Chloraminated	C1	SDX61716.1	Ammonia monooxygenase subunit A	416	31510	7.6	14	8	*Nitrosomonas oligotropha*
		Q8RQ49	Ammonia monooxygenase subunit A	247	31486	6.94	8	5	*Nitrosomonas sp.*
		A0A1B1UFH9	Uncharacterized protein	208	53779	6.89	7	4	*Bradyrhizobium icense*
		AAW48508.1	Ammonia monooxygenase subunit A	185	18295	8.61	7	3	*proteobacterium*
	C2	SDX60482.1	Aerobic hydroxylamine oxidoreductase precursor	742	63912	6.73	24	21	*Nitrosomonas oligotropha*
		SDX61716.1	Ammonia monooxygenase subunit	300	31510	7.60	17	11	*Nitrosomonas oligotropha*
	C3	WP_066646956.1	Hypothetical protein	190	108018	5.69	2	2	*Sphingomonas sp.*
		WP_028179705.1	Polymerase	151	54677	6.51	5	4	*Bradyrhizobium japonicum*
	C4	A0A0K2GIY8	Putative Nitrite oxidoreductase	1034	131657	8.94	18	24	*Nitrospira moscoviensis*
		A0A0S4L679	Nitrite oxidoreductase	807	131698	8.96	15	19	*Candidatus Nitrospira nitrosa*
		WP_066646956.1	Hypothetical protein	127	108018	5.89	2	2	*Sphingomonas species*
	C5	WP_066646956.1	Hypothetical protein	594	108018	5.69	7	11	*Sphingomonas species*
		OHB30721.1	Hypothetical protein	163	106625	4.64	2	2	*Phenylobacterium species*

		A0A0W1I7L3	TonB-dependent receptor	142	11837	4.75	1	3	*Sphingopyxis species*
	C6	WP_066646956.1	Hypothetical protein	455	108018	5.69	5	5	*Sphingomonas species*
		I0G1G5	Putative porin	176	54475	6.07	5	4	*Bradyrhizobium species*
	C7	A0A0K2GIM6	Uncharacterized protein	263	62308	8.35	7	6	*Nitrospira moscoviensis*
		WP_043160929.1	ABC transporter substrate-binding protein	207	66615	8.09	9	5	Unknown species
	C8	A0A0K2GIM6	Uncharacterized protein	295	62308	8.35	7	7	*Nitrospira moscoviensis*
	C9	SDW38467.1	Outer membrane protein (porin)	1073	42240	7.79	37	24	*Nitrosomonas oligotropha*
		A0A0S4L6A0	Nitrite oxidoreductase	677	49888	8.77	31	16	*Candidatus Nitrospira nitrosa*
		F8GKP6	Porin Gram-negative type	612	41863	7.79	20	15	*Nitrosomonas*
	C10	SDW38467.1	Outer membrane protein (porin)	920	42240	7.79	33	22	*Nitrosomonas oligotropha*
		A0A0R3NJ37	Leucine-binding protein	126	43448	8.71	6	3	*Bradyrhizobium retamae*
Ammoniated	A1	A0A0S3AFC8	Hydroxylamine reductase	94	64057	6.12	3	2	*Nitrosomonas ureae*
	A2	F8GL04	Hydroxylamine oxidase	115	63638	6.69	3	2	*Nitrosomonas species*
	A3	C5T1E4	Uncharacterized protein	283	51323	5.73	7	9	*Acidovorax delafieldii*
		WP_051443358.1	Hypothetical protein	222	59150	5.58	4	3	*Curvibacter gracilis*
		A0A0Q6ZFD2	Cell surface protein	167	59440	5.56	5	3	*Acidovorax species*
		S2XNJ4	Uncharacterized protein	103	36297	9.61	3	1	*Delftia acidovorans*

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	A4	A0A0N0K6T2	TonB-dependent receptor	114	105381	8.79	2	2	*Novosphingobium species*
		OHB27609.1	Hypothetical protein	114	107529	4.60	2	2	*Phenylobacterium species*
		A0A0P7A890	Uncharacterized protein	113	109054	4.31	1	2	*Citromicrobium species*
	A5	D8P8B0	Uncharacterized protein	154	61151	8.58	7	3	*Nitrospira defluvii*
	A6	WP_056745241.1	Porin	568	36004	9.38	30	16	*Acidovorax species*
	A7	WP_047149359.1	hypothetical protein	223	13787	10.29	28	3	*Escherichia coli*
		A0A0U1CSI2	Core histone	164	14095	10.10	18	2	*Chlamydia trachomatis*
		WP_017454560.1	Hypothetical protein	161	41946	5.29	9	3	*Pseudoalteromonas sp*
		A0A1C4QRR8	Sugar kinase of the NBD/HSP70 family	110	41573	8.42	2	3	*Streptomyces species*
		WP_043113300.1	Porin	109	35380	8.81	7	2	*Pseudacidovorax intermedius*

Table 8.3: Identified proteins that were identified and their related bacterial species from CDP fall in the range 30 KDa-50 KDa

MW(kDa)	Name of the identified protein for chloraminated reactor	Identified bacterial species
31.5	Ammonia monooxygenase subunit A	*Nitrosomonas oligotropha*
53.8	Uncharacterized protein	*Bradyrhizobium icense*
63.9	Aerobic hydroxylamine oxidoreductase precursor	*Nitrosomonas oligotropha*
54.7	Polymerase	*Bradyrhizobium japonicum*
54.5	Putative porin	*Bradyrhizobium species*
62.3	Uncharacterized protein	*Nitrospira moscoviensis*
42.2	Outer membrane protein (porin)	*Nitrosomonas oligotropha*
49.9	Nitrite oxidoreductase	*Nitrospira nitrosa*
41.9	Porin Gram-negative type	*Nitrosomonas*
43.4	Leucine-binding protein	*Bradyrhizobium retamae*

8.4 Discussion

According to Herath, Torres and Sathasivan (2018), proteins that were responsible for the rapid decay of chloramine fall in 30-50 kDa range. Therefore, among the proteins presented in Table 8.1, protein bands identified between 30 kDa and 50 kDa with high score value can be accountable for the accelerated decay of chloramine in nitrified bulk water samples.

Several proteins such as ammonia monooxygenase (AMO) subunit A, aerobic hydroxylamine oxidoreductase precursor and outer membrane proteins related to *Nitrosomonas* spp. *Nitrosomonas* are identified as the dominant AOB in biofilms and bulk waters of pilot and full scale chloraminated systems identified in this study were also reported by others (Claros et al. 2010; Lipponen et al. 2004; Regan, John M et al. 2003; Regan, John M, Harrington & Noguera 2002).

Enzyme AMO subunit A (band C1 in Table 8.2) is a membrane protein which is responsible for ammonia oxidation and experienced adaptation due to extreme pH environments

(Macqueen & Gubry-Rangin 2016). It is also reported that, ammonia-oxidizing archaea (AOA) in marine systems produces the A and B subunits of AMO in response to ammonia starvation (Qin et al. 2017). As AMO subunit A is produced due to extreme environmental conditions (starvation, extreme pH), it could also be produced due to chloramine disinfection as a stress response. However, AMO sub unit A or related bacteria *(Nitrosomonas europaea)* could not be detected in ammoniated biofilm samples, which may be due to being present in low concentrations rather than the detection limit on MS.

Enzyme hydroxylamine oxidoreductase (band C2 in Table 8.2) is reported from *Nitrosomonas europaea* and catalyses the aerobic oxidation of hydroxylamine to nitrite and nitrate (Bennett et al. 2016). However, nitrate was not produced from nitrite; in the presence of diethyldithiocarbamate, hydroxylamine (a precursor of nitrate) is oxidized to nitrite (Hooper, Terry & Maxwell 1977). As hydroxylamine oxidoreductase does not have any evidence of being produced as a resistance mechanism, further studies of this production against the presence of chloramine need close examination.

Other than *Nitrosomonas* sp., proteins such as putative porin (band C6 in Table 8.2), leucine-binding protein (band C10 in Table 8.2) and polymerase of *Bradyrhizobium* sp were also prominent in the size range of CDP (30-50 kDa) responsible for accelerated chloramine decay.

Protein porins are located in the outer membrane of gram-negative bacteria and one group of gram-positive bacteria (Delcour 2002; Riess et al. 2001). They are also considered as minor proteins (e.g. PhoE and LamB); where synthesis in some cases is strongly induced when they are needed (Macnab 1999; Soto & Hultgren 1999). de María et al. (2007) reported the application of glyphosate herbicide to extracted *Bradyrhizobium* sp. (*Lupinus*) in *Lupinus* plant nodules enhanced the expression of a gene to produce putative protein, which is the first porin identified in *Bradyrhizobium* sp. As putative protein production increases under glyphosate treatment, it could be accountable as a resistance mechanism to glyphosate herbicide. Similarly, outer membrane porin (band C9 in Table 8.2) produced by *Nitrosomonas* could also be related to faster decay of chloramine.

In our protein identification results, the leucine-binding protein is identified as the production/extraction from *Bradyrhizobium retamae*. This protein is a component of the leucine-specific transport system, which is one of the two periplasmic binding protein-dependent transport systems for high-affinity transport of the branched-chain amino acids in

E.coli (Magnusson et al. 2004). However, to establish that this protein production is due to the presence of chloramine, more experiments are needed to justify this argument.

Nitrite oxidoreductase (band C9 & Table 8.2) is an enzyme involved in nitrification found in this current study, originated from the bacterium *Nitrospira nitrosa*. This enzyme is relatively abundant (10-30% of the total protein) and forms closely-packed structures on the inner cytoplasmic surface of the bacterial membrane surface (Spieck et al. 1996). Nevertheless, the relevance to the effect on faster chloramine decay needs in-depth studies.

There are uncharacterised proteins (band C1 and C7 in table 8.2) identified from *Bradyrhizobium icense* and *Nitrospira moscoviensis;* which could be of interest regarding chloramine decay. However, these proteins could not be identified from the current data base search. In summary, bacteria species that were associated with protein identification also comply with bacterial community identification results in chapter 7. The most common autotroph bacterial type in community identification resulted as *Nitrosomonas*. They are Gram-negative betaproteobacteria that can derive all its energy from the oxidation of ammonia to nitrite and live in several places such as soil, sewage, freshwater and the marine. Krishna, Sathasivan and Ginige (2013) reported that betaproteobacteria is one of the dominant species in severely nitrified reactors maintained in the pilot scale reactor set. Additionally, uncharacterized bacterial species from family Bradyrhizobiaceae and order Rizobiales were abundantly identified during bacterial community. One of the bacteria that was identified in CDP; *Bradyrhizobium* sp was also classified under the same order and family.

8.5 Conclusion

The major objective of this study is the separation and identification of active CDP species which are responsible for the gradual loss of chloramine in nitrified water distribution systems. To achieve this aim in this current study, gel electrophoresis followed by mass spectrometry was carried out. The samples used for the experiment were derived from chloraminated and ammoniated reactors. There are several proteins identified in the tested water samples. By identifying specific protein(s) responsible for this chloramine decay as a bio-marker would facilitate early prediction of drinking water condition and subsequent identification of pathogenic bacteria at water storage facilities. Outcomes of such study will be beneficial to all

chloraminated water supply systems in the world. major conclusions made in this study are as follows:

- Various bacterial proteins are present in both chloraminated and ammoniated reactors, but higher in the chloraminated reactor and some of the proteins might be playing a major role in decaying the chloramine.

- With the current bulk water samples used and the methods applied, suspected CDP could not be clearly separated and identified from bulk water.

- Enzyme ammonia monooxygenase subunit A and porin are the main proteins identified in biofilm water samples. The related bacteria; *Nitrosomonas* spp and *Bradyrhizobium sp.* for the identified proteins in chloraminated reactor in the current study, suggest that these bacteria can rapidly grow in chloramine decaying water reactors.

Chapter 9

Silver inhibition on micro-organisms and CDP in nitrified chloraminated water

9.1 Introduction

Micro-organisms may enter the water distribution systems through accidental leaks or faults in process trains and build up over time, these can negatively affect not only the odour and taste of water but also could increase the risk from pathogenic microorganisms. The build-up of biofilm in pipes can also cause problems in the flow-rate and longevity of the system. In order to ensure that the water is safe to drink and use, water utilities employ disinfectants such as chlorine, chloramine, ozone, chlorine dioxide (Lenntech 2004).

Chloramine is being used increasingly as a substitute for chlorine during secondary disinfection of drinking water. The main purpose for the substitution of chlorine to chloramine is that little to no regulated disinfection by-products such as, trihalomethanes and haloacetic acids are formed during chloramine disinfection. Chloramine remains active for much longer within the water system as it takes long time for chloramine to be chemically broken down. Chloramine does not give off any taste or smell and it has been regarded being relatively safe.

A major disadvantage of chloramine is the ability of microbes to decay chloramine relatively faster. Both heterotrophs and autotrophs are known to decay chloramine (Herath, Sathasivan

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& Lam 2015; Sathasivan, Fisher & Kastl 2005; Sathasivan, Fisher & Tam 2008). As chloramine is the combination of chlorine and ammonia, ammonia oxidizing microbes (AOM) oxidise free ammonia to nitrite and then nitrite oxidising bacteria (NOB) form nitrite from nitrate. In the distribution system, high levels of nitrite are usually observed meaning activity of NOB is minimal leaving most nitrite to be oxidized by chloramine. Consequently, the most frequently observed accelerated decay of chloramine in the distribution system is typically accountable for nitrite (AOM). Recent studies of Krishna, Sathasivan and Sarker (2012) reported that SMP which is secreted or released during severe nitrification can harm chloramine drastically. This is further confirmed by Herath, Torres and Sathasivan (2018) as chloramine decaying protein (CDP) produced as a stress response to added chloramine (Chapter 5), decays chloramine in an enhanced manner. Therefore, it is essential to control the nitrification prior to it reaching a severe stage. According to Sathasivan, Fisher and Kastl (2005), Microbial decay can be controlled by adding silver. As per Lalley et al. (2014), silver has proven to be the most efficient and the most commonly inspected disinfecting metal nanoparticle due to its bacterial inactivation properties amongst other metals including copper (Cu), zinc (Zn), iron (Fe), lead (Pb), aluminium (Al) and gold (Au). Silver by itself has inactivation power on planktonic bacteria (Silvestry-Rodriguez et al. 2008).

Antimicrobial effect of Silver against different types of microorganisms has been confirmed in various applications (Cassells et al. 1995; Choi, O et al. 2008; Davies & Etris 1997). A number of microorganisms including bacteria and viruses can be inactivated by the use of copper and silver ions as disinfectants (Sicairos-Ruelas, Gerba & Bright 2019; Yahya, MT et al. 1991). Additionally, the combined effect of copper and silver ions with chlorine results in an improved anti-microbial effect compared to the use of chlorine alone (Sicairos-Ruelas, Gerba & Bright 2019; Yahya, M, Gerba & Rose 1994)

Similarly, Cassells et al. (1995) demonstrated that the combined activity of silver or copper with another disinfectant is highly effective in water disinfection. The no-observed-adverse-effect level (NOAEL) of total lifetime oral intake for human consumption is 10 g for silver; which means that the drinking water contribution to the NOAEL (WHO 2003) is quite insignificant. Higher levels of silver, up to 0.1 mg.L^{-1} (a concentration that yields a total dose over 70 years amounting to half the human NOAEL of 10 g), could then be tolerated without risk to health.

Inhibitory mechanisms of silver on micro-organisms are established by several authors. Ag^+ is believed to deactivate cellular enzymes and cause disruption to membrane permeability, ultimately leading to cell lysis and death due to its sorption to the negatively charged bacterial cell wall (Ratte 1999; Sambhy et al. 2006). The cell death of micro-organisms by Ag^+ is by events of binding onto disulfide or sulfhydryl groups in cell wall proteins (Feng et al. 2000; Slawson et al. 1992) and DNA (Thurman, Gerba & Bitton 1989), thereby disrupting metabolic processes within the cell.

Destruction of DNA due to Ag^+ has been demonstrated via several possible methods. Due to the penetration of Ag^+ into the cells by shrinkage or detachment of cytoplasm membrane from the cell wall, DNA molecules are damaged turning DNA into a condensed form (Feng et al. 2000). Klueh et al. (2000) suggest that Ag^+ enters the cell, intercalate between purine and pyramidine base pairs (which disrupts the hydrogen bonding between the two anti-parallel strands) thus, denaturing the DNA molecule. Other than this, Ag^+ inhibits the respiratory path of sensitive strains, impairs essential enzymes and alters metabolic activity of RNA and DNA with the presence of low chloride ion concentrations (Silver 2003; Starodub & Trevors 1989).

Silver inactivation of proteins (CDP) could lead to prevent the accelerated chloramine decay; thereby, it will assist in managing chloramine above the desired level in distribution systems. Krishna (2012) reported that the application of silver (0.05 mg-Ag.L^{-1}) on nitrified chloraminated bulk water samples largely improved chloramine residual. Moreover, silver was found to be greatly effective in controlling additional chemical decay due to microbial products (CDP) in severely nitrified waters (Sarker 2012). Inactivation of the enzyme activity by interacting with thiol groups in proteins is one of the possible antibacterial mechanisms of protein inhibition by Ag^+. Thiol group is a vital part of proteins responsible for enzymatic activity. Due to the interaction of heavy metals (i.e. Ag^+) with proteins and due to the uniting of thiol groups, the protein could be inactivated (Liau et al. 1997). Pedahzur, Shuval and Ulitzur (1997) explained the destruction of cellular proteins by combined toxic effect of silver and hydrogen peroxide on *E. coli* K-12.

Hence, silver addition is an encouraging way for controlling of chloramine residual in drinking water and it is essential to determine the optimum dose of silver for chloramine decay in chloraminated bulk water. Therefore, 2 sets of batch rechloramination tests were carried out

for nitrified and micro-organisms removed nitrified bulk water samples for the determination of minimum inactivation dosage of $AgNO_3$.

9.2 Materials and Methods

The water samples were collected from the reactor set maintained with 4-5 mg-C.L^{-1} DOC level (detailed in Chapter 3 section 3.3). The total chlorine, nitrogenous species (TAN, nitrite and nitrate) and pH in R-4 (as nitrified reactor) were measured. The results are presented in Table 9.1. TAN is the summation of NH_3-N, NH_4^+-N and nitrogen associated with chloramine.
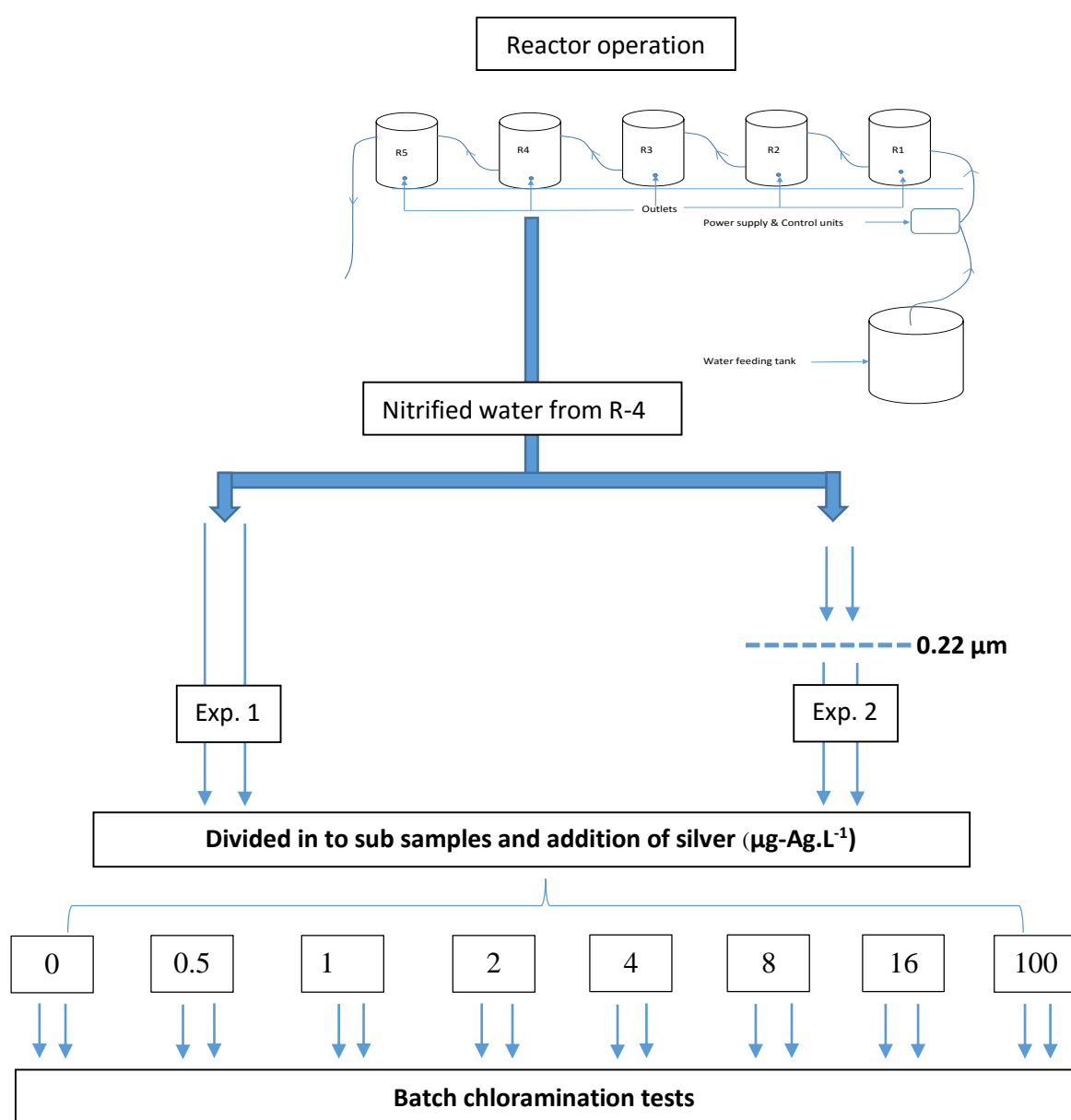
Figure 9.1: Experimental design

9.2.1 First experiment: Addition of silver to the severely nitrified chloraminated water

Batch rechloramination test was carried out for severely nitrified (R-4) water with different concentrations of silver to determine the effective concentration of silver for controlling nitrifying bacteria and decrease of chloramine decay.

A water sample was collected from R-4, divided into 8 subsamples and placed in 500 ml PET bottles. For each of the subsamples, silver was added at 0.5 $\mu g\text{-Ag.L}^{-1}$, 1 $\mu g\text{-Ag.L}^{-1}$, 2 $\mu g\text{-Ag.L}^{-1}$, 4 $\mu g\text{-Ag.L}^{-1}$, 8 $\mu g\text{-Ag.L}^{-1}$, 16 $\mu g\text{-Ag.L}^{-1}$ and 100 $\mu g\text{-Ag.L}^{-1}$. The samples were named as 'SN' with the number for added silver concentration such as, 'SN-0.5'and 'SN-1'. One sample was kept without adding silver as an unprocessed sample (SN-no silver added). Chlorine and ammonia were topped up to 2.5 $mg\text{-Cl}_2\text{.L}^{-1}$ and 0.7 $mg\text{-N.L}^{-1}$, respectively in all subsamples, with the pH value of 8. Afterwards, the samples were incubated in a water bath closed with a lid to maintain a constant temperature (20 $^\circ$C). Total chlorine residual in each sample was periodically monitored.

9.2.2 Second experiment: Addition of silver to the filtered severely nitrified chloraminated water

As an alternative experiment to understand how much silver is needed to control the soluble CDP responsible for the accelerated chloramine decay, different concentrations of silver were added into the filtered severely nitrified bulk water samples collected from R-4.

Microbes were removed by filtering through 0.22 μm sterilised 33 mm syringe driven filter units [housing limit150 psi (10 bars), non-pyrogenic, Millipore Express® PES Membrane, MILLEX®-GP, MIILIPORE IRELAND Ltd. Filtered samples were placed in eight 500 mL PET bottles as sub samples. Silver was added as 0.5 $\mu g\text{-Ag.L}^{-1}$, 1 $\mu g\text{-Ag.L}^{-1}$, 2 $\mu g\text{-Ag.L}^{-1}$, 4 $\mu g\text{-Ag.L}^{-1}$, 8 $\mu g\text{- Ag.L}^{-1}$, 16 $\mu g\text{-Ag.L}^{-1}$ and 100 $\mu g\text{-Ag.L}^{-1}$ in to each sub sample. The samples were named as 'SN filtered' with the number for added silver concentration such as, 'SN filtered-0.5' and 'SN filtered-1'. One sample was kept without adding silver as a control (SN filtered-No silver added).   Initial chemical compositions such as chloramine residual (maintaining Cl-to- TAN ratio of 4:1) and pH were adjusted to 8 in each sample using respective stock chemical solutions. Adjusted chemical parameters were; total chlorine 2.5 $mg.L^{-1}$, TAN 0.7 $mg.L^{-1}$ and nitrite 0.30 $mg\text{-N.L}^{-1}$. Samples were incubated at 20 $^\circ$C. In each sample, total chlorine was monitored over time and the chloramine demand was estimated. At

the time of adding chlorine in to samples, the same dose was applied to ultra-pure water (milli-Q water- Millipore) to ensure that the correct dose was applied. All experiments were done in duplicate. The detailed experimental plan is illustrated in Fig. 9.1.

9.3 Results and discussion

The initial quality of the sample collected reactor set is presented in Table 9.1. The water quality has changed when water travelled from the first reactor (R-1) to the last (R-4). The chemical parameters of R-1 in 4-5 mg-C.L^{-1} DOC level have been described in chapter 5 and chapter 7. Total chlorine residual measured as 1.68 mg-Cl$_2$.L^{-1} in R-1 had decreased to 0.0 mg-Cl$_2$.L^{-1} when water reached R-4. Simultaneously, nitrogenous compounds changed. The pH had also dropped from 7.9 to 7.69. Changes in pH, nitrogenous compounds and chlorine residuals clearly demonstrated the increase in nitrifying bacterial activities between R-1 and R-4 (Wolfe et al. 1990). Furthermore, the difference in nitrite level (0.28 mg-N.L^{-1}) between R-1 and R-4 and low chloramine residual in R-4 revealed that the water in R-4 was severely nitrified as defined by Sathasivan, Fisher and Tam (2008).

Table 9.1: Parameters of the collected water samples before the experiment

Reactors	Total chlorine (mg-Cl$_2$.L^{-1})	TAN (mg.L^{-1})	NOx (mg-N.L^{-1})	Nitrite (mg-N.L^{-1})	Nitrate (mg-N.L^{-1})	pH
R-1	1.68±0.03	0.622±0.010	0.381±0.019	0.005±0.000	0.376±0.008	7.90±0.10
R-2	1.37±0.03	0.44±0.009	0.415±0.021	0.034±0.001	0.381±0.008	7.87±0.10
R-3	0.31±0.03	0.21±0.004	0.509±0.030	0.113±0.004	0.396±0.008	7.75±0.10
R-4[*]	0.09±0.03	0.07±0.001	0.686±0.039	0.284±0.008	0.402±0.008	7.69±0.10
R-5	0.05±0.03	0.05±0.001	0.708±0.041	0.295±0.008	0.413±0.008	7.61±0.10

[*]sample collected reactor for the silver inhibition test

9.3.1 Addition of silver on severely nitrified chloraminated water

Fig. 9.2 explains the chlorine decay profiles within different silver concentrations. The chloramine demand in the first 60 h of the incubation time in SN-no silver added sample is 88%. There were 77% of chloramine demand recorded for both SN-0.5 and SN-1 samples. When the silver concentration increased to 2, 4, 8, 16 and 100 μg-Ag.L^{-1}, 72%, 67%, 62%, 56% and 49% of the added chloramine decayed in the first 60 h, respectively.
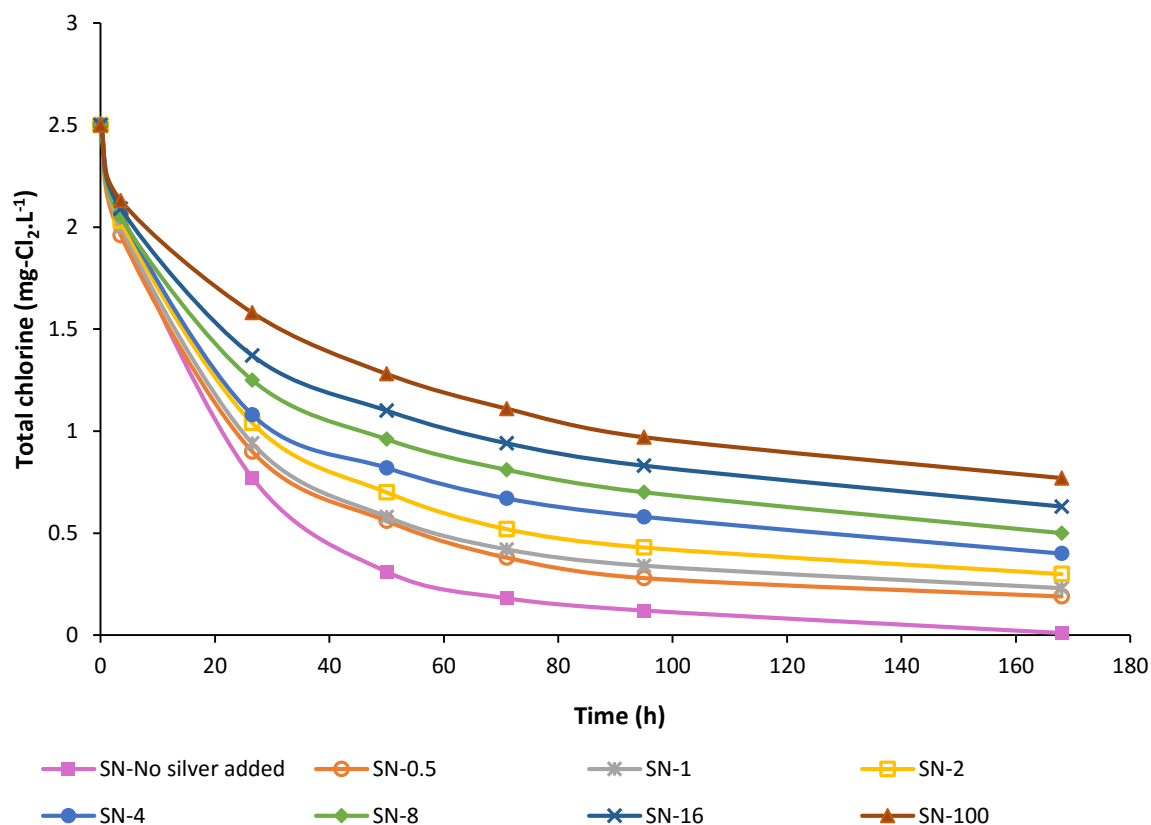
Figure 9.2: Total chlorine decay profiles of not filtered severely nitrified water samples treated with different silver concentrations. Numbers on sample names represent the added silver concentrations in μg-Ag.L^{-1}.

Therefore, according to the results, rapid chloramine loss was observed in the SN-no silver added sample and then the decay rates gradually decreased with increase in the silver concentration. Chloramine decay was well controlled above 2 μg-Ag.L^{-1} of silver concentrations; hence, 2 μg-Ag.L^{-1} silver can be accountable in the effective regulation of chloramine decay.

(a)
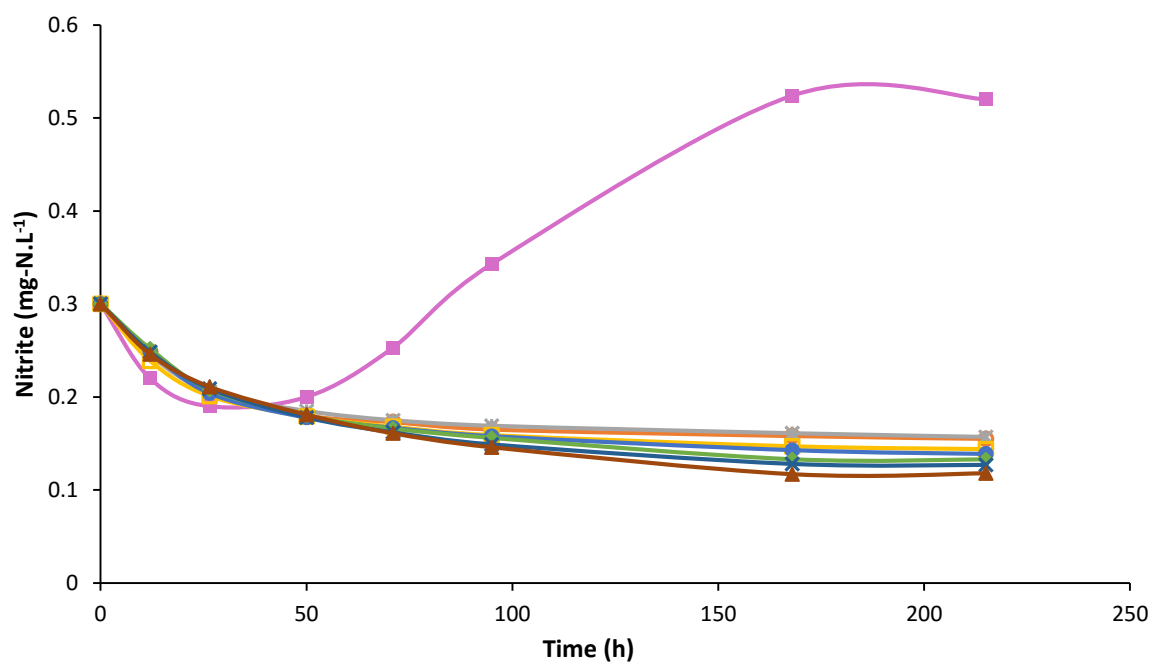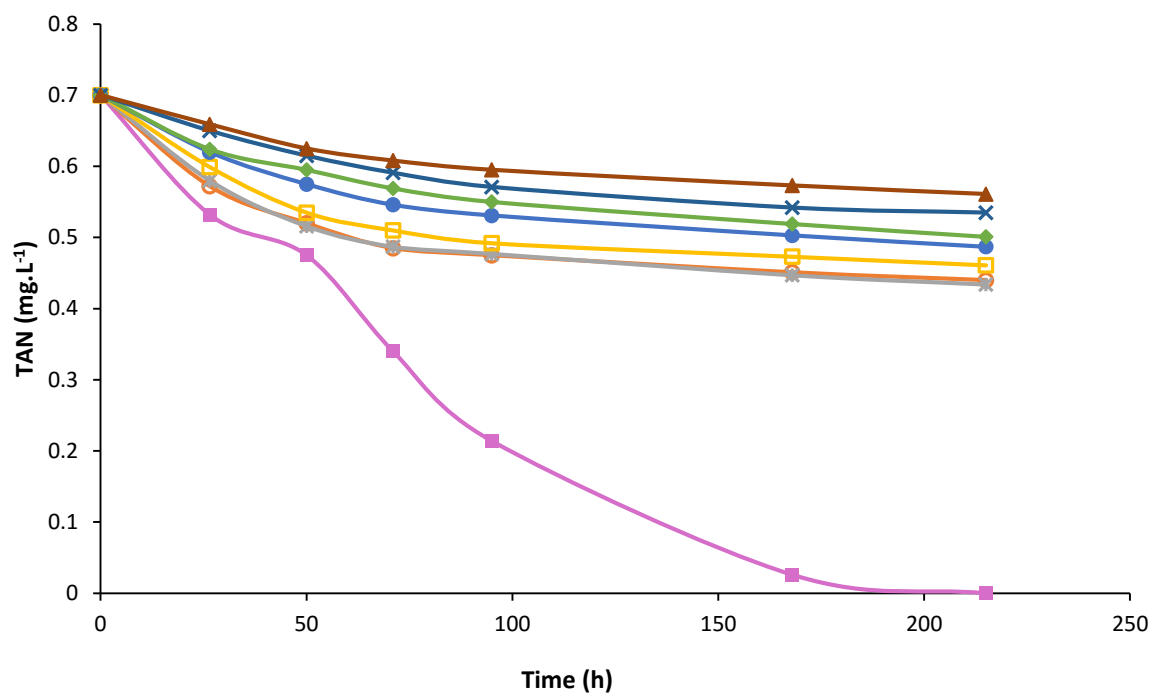
Figure 9.3: Nitrogenous species profiles of not filtered severely nitrified water samples treated with different silver concentrations. Numbers on sample names represent the added silver concentrations in μg-Ag.L^{-1}. 9.3(a): TAN and 9.3(b): Nitrite profiles.

In order to confirm whether the nitrification occurred in the silver added samples, TAN and nitrite results were monitored. Nitrite is only produced by AOM (Sathasivan, Fisher & Tam 2008) in the nitrification processes. In the SN-no silver added sample, the nitrite concentration increased with the reduction of TAN (Fig. 9.3) confirming the occurrence of nitrification. However, according to the nitrite and TAN decay profiles of silver added samples, no gain of nitrite or reduction of TAN were recorded even with the 0.5 µg-Ag.L^{-1} silver concentration. TAN and nitrite levels continuously reduced over the incubation period, the reduction is inversely proportional to the added silver concentration. The results concluded that, while certain levels of chloramine decay may occur in SN silver added samples, nitrification can be completely terminated with minimal (0.5 µg-Ag.L^{-1}) silver addition. The chloramine decay which happens after the addition of such a low concentration of silver could be due to nitrite and CDP which could possibly be bound to the cell wall of the bacteria, in the bulk water and released upon rechloramination. When the silver concentration was increased, these CDP could also be destroyed/inactivated. Nevertheless, further investigation of mechanism and cause is necessary.

9.3.2 Addition of silver for filtered severely nitrified chloraminated water

Different concentrations of silver were added in to filtered severely nitrified water samples collected from R-4. The experiment was conducted as an initial trial for computing the soluble CDP concentration in severely nitrified water samples.

Figure 9.4: Total chlorine decay profiles of filtered (through 0.22 μm) severely nitrified water samples and treated with different silver concentrations. Numbers on sample names represent the added silver concentrations in μg-Ag.L^{-1}.

Chlorine decay profiles with different silver concentrations are represented in Fig. 9.4. The chloramine loss for the SN-filtered-no silver added sample was 66% in the first 40 h of incubation time. Chloramine demand for other samples were 54%, 52%, 46%, 44%, 40%, 38% and 39% for 0.5, 1, 2, 4, 8, 16 and 100 μg-Ag.L^{-1} silver concentrations, respectively. The highest decay rate observed was for the nitrified filtered sample (SN-filtered-no silver added) where it was assumed to have the highest CDP as there was no addition of silver. Afterwards, CDP, the protein responsible for chloramine decay, may have been inactivated by the applied silver; the reduced decay rates were being observed in comparison to increasing silver concentrations. However, after 8 μg-Ag.L^{-1} of silver addition, these patterns could no longer be observed to be resulting in similar chloramine loss. Similarly, when the added silver concentrations were higher than 2 μg-Ag.L^{-1}, the decay rate profiles did not change exceptionally. This may hint that all of the protein present in the tested sample had already been inactivated by 8 μg-Ag.L^{-1} of silver. Therefore, it could be concluded that increasing of silver concentrations beyond this level would not result in any changes in chloramine decay profiles.

(a)
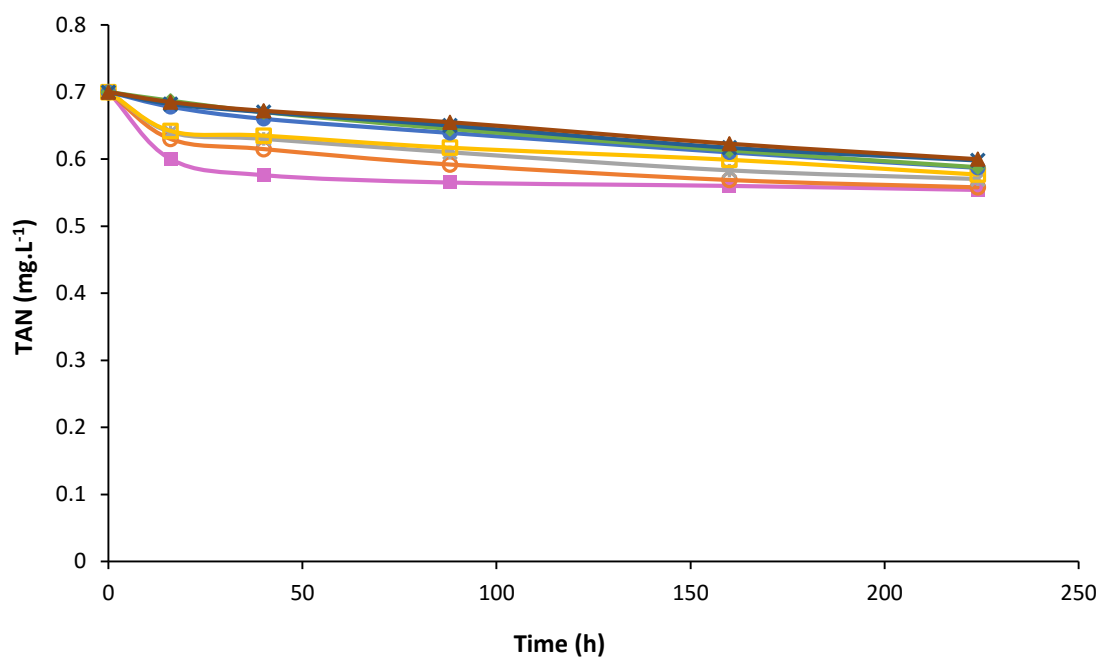
(b)

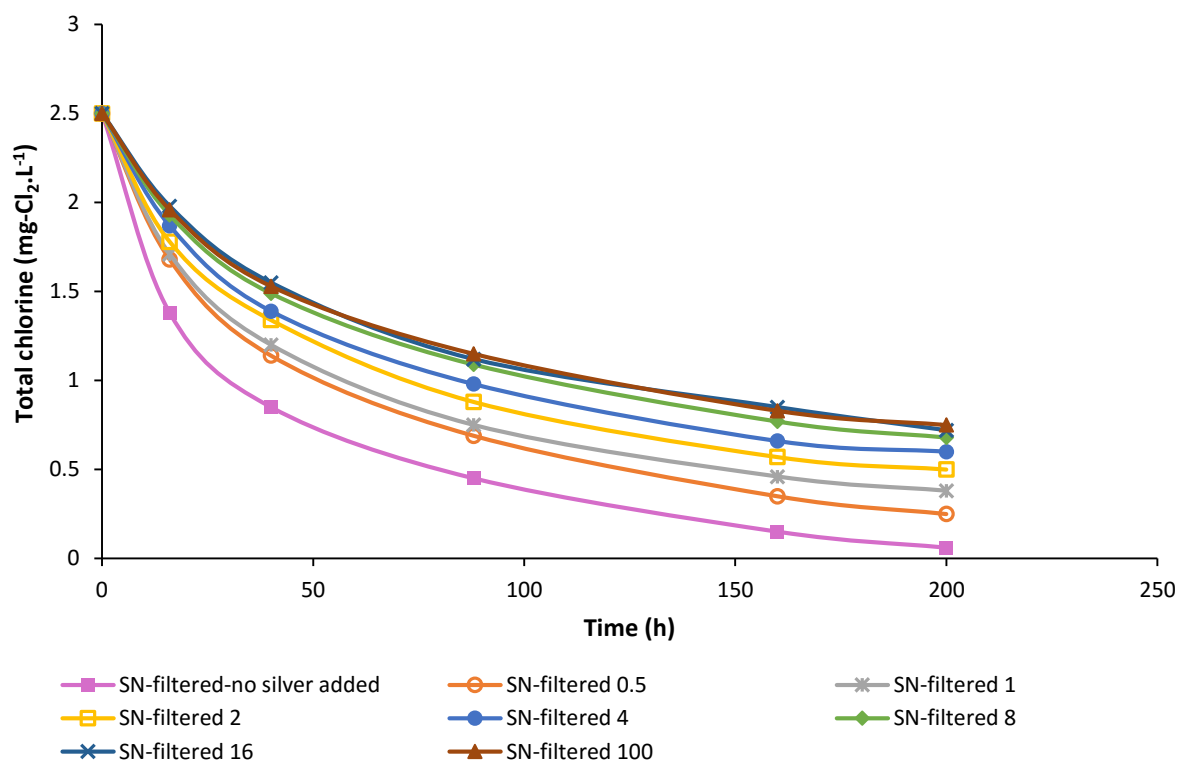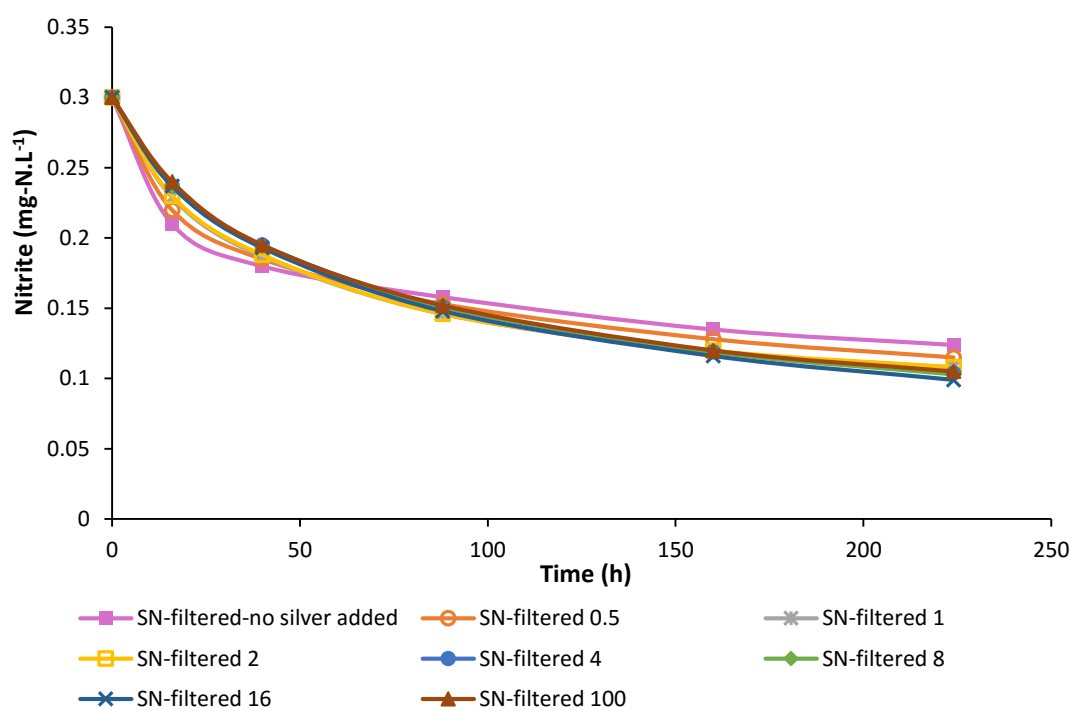**Figure 9.5:** Nitrogenous species profiles of filtered (through 0.22 μm) severely nitrified water samples treated with different silver concentrations. Numbers on sample names represent the added silver concentrations in μg-Ag.L^{-1}. 9.5(a): TAN and 9.5(b): Nitrite profiles.

The highest TAN drop (Fig. 9.5a) was observed in no silver added sample. However, after the first 40 h, the TAN decay profile in the sample is almost constant until the end of the experimental period (i.e., 224 h). In the 0.5 µg-Ag.L^{-1} to 2 µg-Ag.L^{-1} silver added samples, the observed TAN drops were almost similar in the first 40 h, and then continuously reduced while maintaining minor gaps in-between samples.

There are no initial drops, nor prominent decays experienced in other silver added samples (4-100 µg-Ag.L^{-1}) in terms of TAN profiles. Without showing major differences between samples, the nitrite levels reduced continuously over the experimental period (Fig. 9.5b). However, no silver added and 0.5 µg-Ag.L^{-1} silver added samples display faster drops in the first 40 h, and then their decay levels become slower than the other samples; which had not demonstrated a noticeable initial drop. Similar to silver added samples, the TAN and nitrite drop in the no silver added filtered sample had the same pattern as that in the no silver added not filtered sample (Fig. 9.3) in the first 40 h.

Considering the faster chloramine decay results of no silver added sample, it can establish the presence of CDP in filtered nitrified samples. And it is also confirmed as these decay profiles were not due to the microbial presence/activity. Additionally, as there were no differences in the decay profiles observed in more than 2 µg-Ag.L^{-1} silver added samples, all CDP must have been occupied by the silver which was added at more than 2 µg-Ag.L^{-1}. Therefore, with 2 µg-Ag.L^{-1} of silver; the CDP can be completely eliminated/deactivated in the tested nitrified water samples. In conclusion, this method can be used to find the CDP concentration in a given nitrified water sample.

Biologically, silver is an unnecessary, non-beneficial element to humans. There are no scientific studies to indicate adverse health due to its presence in drinking water from natural sources. However, when ingested and absorbed, silver is held indefinitely within tissue, particularly skin, eyes, and mucous membranes. Skin discoloration is a cosmetic effect related to silver ingestion (WHO 2004).

The U.S. Environmental Protection Agency has set a non-enforceable secondary standard for silver because of its ability to cause aesthetic discolorations of the skin. This mirrors the World Health Organization set a guideline also at 0.1 µg-Ag.L^{-1} over a lifetime of exposure in drinking water. The Secondary Maximum Contaminant Level (SMCL) for silver is 0.10 µg-Ag.L^{-1}. Our findings concluded, with 2 µg-Ag.L^{-1} silver can inhibit the nitrification and chloramine decaying protein effectively. Therefore, applying silver in such concentrations to control the

nitrification and related issues is far below than the guideline recommendations for silver exposure.

9.4 Conclusion

Different silver concentrations were applied in to the nitrified not filtered and nitrified filtered samples to determine the optimum inactivation concentration which obstruct accelerated chloramine decay. The conclusions made are as follows;

- Addition of silver greatly reduced the accelerated chloramine decay in severely nitrified bulk water samples.

- $2\ \mu g\text{-Ag.L}^{-1}$ silver is effective controlling nitrifying bacteria and completely terminate the nitrification process.

- Optimal concentration of silver controlling CDP can be concluded as $2\ \mu g\text{-Ag.L}^{-1}$ and complete inhibition happened around $8\ \mu g\text{-Ag.L}^{-1}$.

Chapter 10

Conclusions and Recommendations

10.1 Conclusions

This research has been undertaken to maintain necessary chloramine residuals throughout the chloraminated water distribution systems by understanding microbial factors accelerating chloramine decay. It was recently found out soluble microbial products (SMP) suspected as proteins present in the nitrified water from distribution systems accelerate chloramine decay. Therefore, it is essential to investigate the factors promoting SMP production, and characterise and identify SMP. Knowing the essential characteristics, it is important to evaluate appropriate control mechanisms so that impact of SMP can be minimised. The conclusions made in this study are as detailed below.

Initial trials of my previous study indicated that the dissolved organic carbon (DOC) level substantially controlled chloramine decay. Those results also indicated that chloramine decaying SMP (cSMP) could contain proteins and have a molecular size of 30–50 kDa, and it behaves in a similar manner to that observed in severely nitrifying water. Hence, this cSMP is termed as chloramine decaying proteins (CDP). Therefore, four chloraminated lab-scale reactor sets were operated (fed) with water of different DOC levels (0-8 mg-C.L^{-1} DOC) to understand the effect of DOC level on CDP impact. In parallel, the bacterial community variations were also analysed on the same reactor sets. With the increase of DOC level, the impact of CDP on

severely nitrifying samples generally decreased. Additionally, all chemical chloramine decay in CDP containing samples can be explained by nitrite oxidation and auto-decomposition catalysed by CDP.

The DOC variation of feed water did not significantly affect the microbial community present in reactor sets operated with different levels of DOC contained chloraminated water samples and the similarities observed between reactors from each reactor set are very high. The ammonia oxidising bacterial (AOB) activity does not correlate with the decay rate co-efficient in individual reactor sets with varying DOC levels. However, AOB activity is the highest in the reactor set with lowest DOC level. As there was no single bacterial community that could be directly correlated with chloramine decay, mixed culture could be responsible for the chloramine decay in each reactor set. However, there were several bacterial types identified in samples that are already known to produce extra polymeric substances (EPS); which can also be considered as accountable for CDP as well. Some of the EPS producing bacterial types detected were *Mycobacterium*, *Methylobacterium*, AOB, *Bradyrhizobium sp*. and family Sphingomonadaceae.

Two nitrified reactors with chloramine (chloraminated reactor) and ammonia (ammoniated reactor) were operated with nutrient added ultra-pure water to understand how microbes destroy chloramine and survive by producing CDP. The nitrified conditions were initiated with identical seed water samples collected from a severely nitrified reactor. The bacterial community variations were also analysed to identify the responsible microbes producing CDP. There are three types of CDP that can be presented in the nitrified water samples such as, soluble, insoluble and microbial attached. Even though both reactors were in severely nitrified conditions, the soluble CDP concentration increased in the chloraminated reactor, but decreased in the ammoniated reactor. However, the application of 1.5 mg.L^{-1} chloramine for 1.5 h induced the release/production of CDP from the biomass collected from the chloraminated reactor, but not for the biomass collected from the ammoniated reactor. Furthermore, bulk water microbes in the ammoniated reactor could not bounce back from the introduction of high dose of chloramine (3-3.4 mg-$Cl_2.L^{-1}$), possibly due to the absence of their defence mechanism (CDP producing ability). Bulk water microbes from the chloraminated reactor recovered quickly by destroying the chloramine due to their ability to produce additional CDP as a stress response, possibly due to the presence of their defence mechanism.

Conclusions made from bacterial community analysis on the chloraminated and ammoniated reactors are as follows. Absence of high DOC level in both the reactors does not interfere with the heterotrophic bacterial community and therefore; Alphaproteobacteria (heterotrophs) is the dominant bacterial class. Consequently, nitrifiers could provide the substrate for heterotrophic bacterial growth and survival by releasing/producing CDP. Major variances in bacterial community composition and diversity could not be seen in the chloraminated, ammoniated and seed water collected reactors. However, the EPS producing bacterial strains (AOB, *Bradyrhizobium sp*. and family Sphingomonadaceae) identified in chloraminated reactors are suspected to be responsible for CDP production.

Herath (2014) has evidenced more than 25 proteins in nitrified water samples displaying faster chloramine decay; that can pass through 50 KDa membrane and retain on 30 kDa cut off membrane. It was hard to separately identify specific CDP from within these proteins and the concentration of proteins was also not sufficient for further identification. Therefore, protein separation and identification were examined on chloraminated and ammoniated reactors to narrow down identified protein numbers by increasing protein concentration. While various bacterial proteins were present in both chloraminated and ammoniated reactors, they were higher in the chloraminated reactor and therefore; some of the proteins might be playing a major role in decaying chloramine. With current bulk water samples used and methods applied, the suspected CDP could not be clearly separated and identified from bulk water. Therefore, further investigations were conducted using biofilm samples that consisted of more microbes and therefore, more CDP. Enzyme ammonia monooxygenase subunit A and porin are the main proteins identified in biofilm water samples in the chloraminated reactor. The related bacteria for these identified proteins are *Nitrosomonas* spp and *Bradyrhizobium sp.* respectively. Therefore, the current study suggests that these bacteria can rapidly grow in chloramine decaying water reactors.

Furthermore, with the intension of providing safe water for consumers, silver inhibition on nitrifiers and CDP was investigated. Addition of silver greatly reduces the accelerated chloramine decay in severely nitrified bulk water samples. $2 \mu g\text{-}Ag.L^{-1}$ silver is sufficient and effective in controlling nitrifying bacteria and completely terminates the nitrification process. Therefore the optimal concentration of silver to control CDP can be concluded as $2 \mu g\text{-}Ag.L^{-1}$ and complete inhibition occurs around $8 \mu g\text{-}Ag.L^{-1}$.

10.2 Recommendations for future studies

Chloramination is practiced as one of the disinfection method in drinking water distribution systems. However, chloramine decays due to chemical and biological reasons. Previously, only nitrification was considered as microbial chloramine decay, but our team questioned the traditional belief and developed various mechanisms of microbial chloramine decay. Heterotrophic bacteria and microbial products secreted by micro-organisms living in the chloraminated systems have been found as responsible for major part of this accelerated chloramine decay. As microbial decay is more significant than the chemical decay of chloramine, it is comprehensively investigated under the ARC linkage project (LP160100909). SMP is the most important segment of this microbial chloramine decay. Therefore, my study was mainly focused on understanding the nature, its control and which microbes produced the SMP. I will therefore focus on how the experiments could be further developed.

In the pursuit of my experiments, I have succeeded in identifying SMP as protein of size 30-50 KDa and named it as chloramine decaying protein (CDP) but, the actual nature of protein is still not known. In identifying the bacteria responsible, various experiments were conducted by creating an environment that may encourage their growth. Reactors were maintained with different DOC level in the feed water and reactors with stress condition-chloramine (chloraminated reactor) and ammonia (ammoniated reactor). Later, MiSeq was performed on these reactors. In my experiments, stress was evidenced as one of the cause of producing CDP. When DOC concentration was higher, the CDP was present at low concentration. The identification of a particular bacteria was still not possible since all bacteria were present in most environment. However, protein analysis indicted number of bands out of which *Nitrosomonas oligotropha* and *Bradyrhizobium* sp. were suspected to contain the identified proteins.

As the continuation of my work, creating stress conditions with different chloramine levels such as 1, 1.5 and 2 .0 mg.L^{-1} with seeds from original rector. This possibly could narrow down the identification process. Furthermore, as Miseq only does community analysis and very high diversity witnessed in the microbial communities within the same sample evaluated, Hiseq could be verified. Isotopic probing and imaging mass spectrometry in microbial community identification will facilitate of identifying the fate of C and N.

I have already tested silver inhibition and demonstrated 2 μg-Ag.L^{-1} can inhibit the accelerated chloramine decay. Therefore, effect of the other inhibitory heavy metals (e.g., Cd, Pb, Zn, Mn and Ni) should be investigated.

Evaluating the impact of breakpoint chlorination and rechloramination will lead to a new understanding in managing chloramine residuals. Furthermore, chloramine decay mechanisms can be predicted, identified and prevented by developing a suitable model by incorporating critical issues such CDP and heterotrophs on chloramine decay.

References

Abd-Alla, M 1994, 'Phosphodiesterase and phosphotriesterase in Rhizobium and Bradyrhizobium strains and their roles in the degradation of organophosphorus pesticides', *Letters in applied microbiology*, vol. 19, no. 4, pp. 240-3.

Adham, S & Gramith, K 2003, 'Development of an MF/UF knowledge base', in *2003 AWWA Annual Conference*, p. 2003.

Alleman, JE, Keramida, V & Pantea-Kiser, L 1987, 'Light induced Nitrosomonas inhibition', *water research*, vol. 21, no. 4, pp. 499-501.

Alleman, JE & Preston, K 1991, 'Behavior and physiology of nitrifying bacteria', in *Proceedings of the second annual conference on commercial aquaculture, CES*, vol. 240, pp. 1-13.

Allen, MJ, Edberg, SC & Reasoner, DJ 2004, 'Heterotrophic plate count bacteria—what is their significance in drinking water?', *International journal of food microbiology*, vol. 92, no. 3, pp. 265-74.

Amy, GL, Sierka, RA, Bedessem, J, Price, D & Tan, L 1992, 'Molecular size distributions of dissolved organic matter', *Journal (American Water Works Association)*, pp. 67-75.

APHA, A 1998, 'Wpcf', *Standard methods for the examination of water and wastewater*, vol. 20.

Aune, KC & Tanford, C 1969, 'Thermodynamics of the denaturation of lysozyme by guanidine hydrochloride. II. Dependence on denaturant concentration at 25', *Biochemistry*, vol. 8, no. 11, pp. 4586-90.

AWW 2006, *Fundamentals and Control of Nitrification in Chloraminated Drinking Water Distribution Systems*, American Water Works Association.

Ayarza, JM, Mazzella, MA & Erijman, L 2015, 'Expression of stress-related proteins in Sediminibacterium sp. growing under planktonic conditions', *Journal of basic microbiology*, vol. 55, no. 9, pp. 1134-40.

Barker, DJ & Stuckey, DC 1999, 'A review of soluble microbial products (SMP) in wastewater treatment systems', *water research*, vol. 33, no. 14, pp. 3063-82.

Bartram, J, Cotruvo, J, Exner, M, Fricker, C & Glasmacher, A 2003, *Heterotrophic plate counts and drinking-water safety*, IWA publishing.

Bartram, J, Cotruvo, J, Exner, M, Fricker, C & Glasmacher, A 2004, 'Heterotrophic plate count measurement in drinking water safety management: report of an Expert Meeting Geneva, 24–25 April 2002', *International journal of food microbiology*, vol. 92, no. 3, pp. 241-7.

Bennett, K, Sadler, NC, Wright, AT, Yeager, C & Hyman, MR 2016, 'Activity-based protein profiling of ammonia monooxygenase in Nitrosomonas europaea', *Applied and Environmental Microbiology*, pp. AEM. 03556-15.

Blankert, B, Betlem, BH & Roffel, B 2007, 'Development of a control system for in-line coagulation in an ultrafiltration process', *Journal of membrane science*, vol. 301, no. 1-2, pp. 39-45.

Bock, E, Koops, H-P, Ahlers, B, Harms, H, Balows, A, Truper, H, Dworkin, M, Harder, W & Schleifer, K 1992, 'Oxidation of inorganic nitrogen compounds as energy source', *The Prokaryotes.*, no. Ed. 2, pp. 414-30.

Bridier, A, Briandet, R, Thomas, V & Dubois-Brissonnet, F 2011, 'Resistance of bacterial biofilms to disinfectants: a review', *Biofouling*, vol. 27, no. 9, pp. 1017-32.

Burtscher, MM, Zibuschka, F, Mach, RL, Lindner, G & Farnleitner, AH 2009, 'Heterotrophic plate count vs. in situ bacterial 16S rRNA gene amplicon profiles from drinking water reveal completely different communities with distinct spatial and temporal allocations in a distribution net', *Water Sa*, vol. 35, no. 4.

Campinas, M & Rosa, MJ 2010, 'Assessing PAC contribution to the NOM fouling control in PAC/UF systems', *water research*, vol. 44, no. 5, pp. 1636-44.

Caporaso, JG, Kuczynski, J, Stombaugh, J, Bittinger, K, Bushman, FD, Costello, EK, Fierer, N, Pena, AG, Goodrich, JK & Gordon, JI 2010, 'QIIME allows analysis of high-throughput community sequencing data', *Nature methods*, vol. 7, no. 5, p. 335.

Cappelletti, M, Frascari, D, Zannoni, D & Fedi, S 2012, 'Microbial degradation of chloroform', *Applied microbiology and biotechnology*, vol. 96, no. 6, pp. 1395-409.

Carlson, KH & Amy, GL 2000, 'The importance of soluble microbial products (SMPs) in biological drinking water treatment', *water research*, vol. 34, no. 4, pp. 1386-96.

Carson, LA, Petersen, NJ, Favero, MS & Aguero, SM 1978, 'Growth characteristics of atypical mycobacteria in water and their comparative resistance to disinfectants', *Applied and Environmental Microbiology*, vol. 36, no. 6, pp. 839-46.

Cassells, J, Yahya, M, Gerba, CP & Rose, JB 1995, 'Efficacy of a combined system of copper and silver and free chlorine for inactivation of Naegleria fowleri amoebas in water', *Water Science and Technology*, vol. 31, no. 5-6, p. 119.

Chapman, JS 2003, 'Disinfectant resistance mechanisms, cross-resistance, and co-resistance', *International biodeterioration & biodegradation*, vol. 51, no. 4, pp. 271-6.

Chistoserdova, L, Jenkins, C, Kalyuzhnaya, MG, Marx, CJ, Lapidus, A, Vorholt, JA, Staley, JT & Lidstrom, ME 2004, 'The enigmatic planctomycetes may hold a key to the origins of methanogenesis and methylotrophy', *Molecular biology and evolution*, vol. 21, no. 7, pp. 1234-41.

Cho, M-H, Lee, C-H & Lee, S 2006, 'Effect of flocculation conditions on membrane permeability in coagulation–microfiltration', *Desalination*, vol. 191, no. 1-3, pp. 386-96.

Choi, I-H, Lee, S-m, Kim, I-C, Min, B-R & Lee, K-H 2007, 'Effect of new photocatalytic coagulant on NF membrane fouling', *Industrial & engineering chemistry research*, vol. 46, no. 8, pp. 2280-5.

Choi, O, Deng, KK, Kim, N-J, Ross Jr, L, Surampalli, RY & Hu, Z 2008, 'The inhibitory effects of silver nanoparticles, silver ions, and silver chloride colloids on microbial growth', *water research*, vol. 42, no. 12, pp. 3066-74.

Choi, YH, Kim, HS & Kweon, JH 2008, 'Role of hydrophobic natural organic matter flocs on the fouling in coagulation-membrane processes', *Separation and Purification Technology*, vol. 62, no. 3, pp. 529-34.

Claros, J, Jiménez, E, Aguado, D, Ferrer, J, Serralta, J, Borrás, L & Seco, A 2010, 'Short-term effect of ammonia concentration and salinity on activity of ammonia oxidizing bacteria', *Water Science & Technology*, vol. 61, no. 12.

Coligan, J, Dunn, B, Speicher, D & Wingfield, P 1995, 'Current protocols in protein science. 1995', *New York: Weily Google Scholar*.

Cooper, GM 2000, 'The central role of enzymes as biological catalysts', in Sinauer Associates.

Covert, TC, Rodgers, MR, Reyes, AL & Stelma, GN 1999, 'Occurrence of nontuberculous mycobacteria in environmental samples', *Applied and Environmental Microbiology*, vol. 65, no. 6, pp. 2492-6.

Critchley, M, Cromar, N, McClure, N & Fallowfield, H 2003, 'The influence of the chemical composition of drinking water on cuprosolvency by biofilm bacteria', *Journal of applied microbiology*, vol. 94, no. 3, pp. 501-7.

Cunliffe, DA 1991, 'Bacterial nitrification in chloraminated water supplies', *Applied and Environmental Microbiology*, vol. 57, no. 11, pp. 3399-402.

Daifullah, A, Girgis, B & Gad, H 2004, 'A study of the factors affecting the removal of humic acid by activated carbon prepared from biomass material', *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 235, no. 1-3, pp. 1-10.

Daims, H, Lebedeva, EV, Pjevac, P, Han, P, Herbold, C, Albertsen, M, Jehmlich, N, Palatinszky, M, Vierheilig, J & Bulaev, A 2015, 'Complete nitrification by Nitrospira bacteria', *Nature*, vol. 528, no. 7583, p. 504.

Davies, RL & Etris, SF 1997, 'The development and functions of silver in water purification and disease control', *Catalysis Today*, vol. 36, no. 1, pp. 107-14.

de María, N, Guevara, A, Serra, MT, García-Luque, I, González-Sama, A, de Lacoba, MG, de Felipe, MR & Fernández-Pascual, M 2007, 'Putative porin of Bradyrhizobium sp.(Lupinus) bacteroids induced by glyphosate', *Applied and Environmental Microbiology*, vol. 73, no. 16, pp. 5075-82.

Delcour, AH 2002, 'Structure and function of pore-forming b-barrels from bacteria', *J. Mol. Microbiol. Biotechnol*, vol. 4, no. 1, pp. 1-10.

Dlamini, S, Haarhoff, J, Mamba, B & Van Staden, S 2013, 'The response of typical South African raw waters to enhanced coagulation', *Water Science and Technology: Water Supply*, vol. 13, no. 1, pp. 20-8.

Dodds, MG, Grobe, KJ & Stewart, PS 2000, 'Modeling biofilm antimicrobial resistance', *Biotechnology and bioengineering*, vol. 68, no. 4, pp. 456-65.

Dolgikh, D, Gilmanshin, R, Brazhnikov, E, Bychkova, V, Semisotnov, G, Venyaminov, SY & Ptitsyn, O 1981, 'α-lactalbumin: compact state with fluctuating tertiary structure?', *FEBS letters*, vol. 136, no. 2, pp. 311-5.

Dolinšek, J, Lagkouvardos, I, Wanek, W, Wagner, M & Daims, H 2013, 'Interactions of nitrifying bacteria and heterotrophs: identification of a Micavibrio-like putative predator of Nitrospira spp', *Appl. Environ. Microbiol.*, vol. 79, no. 6, pp. 2027-37.

Dong, L, Liu, W, Jiang, R & Wang, Z 2014, 'Physicochemical and porosity characteristics of thermally regenerated activated carbon polluted with biological activated carbon process', *Bioresource technology*, vol. 171, pp. 260-4.

Douterelo, I, Fish, K & Boxall, J 2018, 'Succession of bacterial and fungal communities within biofilms of a chlorinated drinking water distribution system', *water research*, vol. 141, pp. 74-85.

Duirk, SE, Gombert, B, Croué, J-P & Valentine, RL 2005, 'Modeling monochloramine loss in the presence of natural organic matter', *water research*, vol. 39, no. 14, pp. 3418-31.

Dussert, B 1994, 'The biological activated carbon process for water purification', *Water Engineering and Management*, vol. 141, pp. 22-4.

Duursma, EK 1963, 'The production of dissolved organic matter in the sea, as related to the primary gross production of organic matter', *Netherlands Journal of Sea Research*, vol. 2, no. 1, pp. 85-94.

Edgar, RC 2010, 'Search and clustering orders of magnitude faster than BLAST', *Bioinformatics*, vol. 26, no. 19, pp. 2460-1.

Elechiguerra, JL, Burt, JL, Morones, JR, Camacho-Bragado, A, Gao, X, Lara, HH & Yacaman, MJ 2005, 'Interaction of silver nanoparticles with HIV-1', *Journal of nanobiotechnology*, vol. 3, no. 1, p. 6.

Emtiazi, F, Schwartz, T, Marten, SM, Krolla-Sidenstein, P & Obst, U 2004, 'Investigation of natural biofilms formed during the production of drinking water from surface water embankment filtration', *water research*, vol. 38, no. 5, pp. 1197-206.

Fabris, R, Chow, CW & Drikas, M 2012, 'Comparison of coagulant type on natural organic matter removal using equimolar concentrations', *Journal of Water Supply: Research and Technology-Aqua*, vol. 61, no. 4, pp. 210-9.

Feng, QL, Wu, J, Chen, G, Cui, F, Kim, T & Kim, J 2000, 'A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus', *Journal of biomedical materials research*, vol. 52, no. 4, pp. 662-8.

Ferguson, B, DiGiano, F & Chandran, K 2005, 'Impact of Temporary Switches from Monochloramine to Free Chlorine on Water Quality in Distribution System', in *Annual Conference of AWWA, San Francisco, California., AWWA*.

Filik, J & Stone, N 2009, 'Investigation into the protein composition of human tear fluid using centrifugal filters and drop coating deposition Raman spectroscopy', *Journal of Raman Spectroscopy*, vol. 40, no. 2, pp. 218-24.

Fisher, I, Sathasivan, A, Chuo, P & Kastl, G 2009, 'Effects of stratification on chloramine decay in distribution system service reservoirs', *water research*, vol. 43, no. 5, pp. 1403-13.

Flemming, H-C & Wingender, J 2010, 'The biofilm matrix', *Nature reviews microbiology*, vol. 8, no. 9, p. 623.

Fonte, ES, Amado, AM, Meirelles-Pereira, F, Esteves, FA, Rosado, AS & Farjalla, VF 2013, 'The combination of different carbon sources enhances bacterial growth efficiency in aquatic ecosystems', *Microbial ecology*, vol. 66, no. 4, pp. 871-8.

Ford, DL 1981, 'Troubleshooting and Control of Nitrification Wastewater Treatment Systems', *AIChE Nat. Mtg, Boston, Portland, Chicago, 1980*, vol. 1981, no. 9.

Furuhata, K, Kato, Y, Goto, K, Hara, M, Yoshida, Si & Fukuyama, M 2006, 'Isolation and identification of Methylobacterium species from the tap water in hospitals in Japan and their antibiotic susceptibility', *Microbiology and immunology*, vol. 50, no. 1, pp. 11-7.

Furuhata, K & Koike, K 1993, 'Isolation of Methylobacterium spp. from drinking tank-water and resistance of isolates to chlorine', *[Nihon koshu eisei zasshi] Japanese journal of public health*, vol. 40, no. 11, pp. 1047-53.

Furumai, H & Rittmann, B 1992, 'Advanced modeling of mixed populations of heterotrophs and nitrifiers considering the formation and exchange of soluble microbial products', *Water Science and Technology*, vol. 26, no. 3-4, pp. 493-502.

Gagnon, G, Rand, J, O'leary, K, Rygel, A, Chauret, C & Andrews, R 2005, 'Disinfectant efficacy of chlorite and chlorine dioxide in drinking water biofilms', *water research*, vol. 39, no. 9, pp. 1809-17.

Gallagher, SR 2007, 'One-dimensional SDS gel electrophoresis of proteins', *Current protocols in toxicology*, vol. 32, no. 1, pp. A. 3F. 1-A. 3F. 38.

García, I & Moreno, L 2009, 'Use of GAC after enhanced coagulation for the removal of natural organic matter from water for purification', *Water Science and Technology: Water Supply*, vol. 9, no. 2, pp. 173-80.

Ghernaout, D, Ghernaout, B & Naceur, MW 2011, 'Embodying the chemical water treatment in the green chemistry—A review', *Desalination*, vol. 271, no. 1-3, pp. 1-10.

Gomez-Alvarez, V, Pfaller, S, Pressman, J, Wahman, D & Revetta, R 2016, 'Resilience of microbial communities in a simulated drinking water distribution system subjected to disturbances: role of conditionally rare taxa and potential implications for antibiotic-resistant bacteria', *Environmental Science: Water Research & Technology*, vol. 2, no. 4, pp. 645-57.

Gonçalves, M, Guerreiro, MC, Ramos, PH, de Oliveira, LCA & Sapag, K 2013, 'Activated carbon prepared from coffee pulp: potential adsorbent of organic contaminants in aqueous solution', *Water Science and Technology*, vol. 68, no. 5, pp. 1085-90.

Goslan, EH, Gurses, F, Banks, J & Parsons, SA 2006, 'An investigation into reservoir NOM reduction by UV photolysis and advanced oxidation processes', *Chemosphere*, vol. 65, no. 7, pp. 1113-9.

Goslan, EH, Krasner, SW, Bower, M, Rocks, SA, Holmes, P, Levy, LS & Parsons, SA 2009, 'A comparison of disinfection by-products found in chlorinated and chloraminated drinking waters in Scotland', *water research*, vol. 43, no. 18, pp. 4698-706.

Gray, SR, Dow, N, Orbell, J, Tran, T & Bolto, B 2011, 'The significance of interactions between organic compounds on low pressure membrane fouling', *Water Science and Technology*, vol. 64, no. 3, pp. 632-9.

Grunditz, C & Dalhammar, G 2001, 'Development of nitrification inhibition assays using pure cultures of Nitrosomonas and Nitrobacter', *water research*, vol. 35, no. 2, pp. 433-40.

Haas, C 2000, 'Disinfection. RD Letterman, editor', *Water Quality & Treatment, A Handbook of Community Water Supplies, 5th edition. McGraw-Hill, Inc., New York.*

Habimana, O, Steenkeste, K, Fontaine-Aupart, M-P, Bellon-Fontaine, M-N, Kulakauskas, S & Briandet, R 2011, 'Diffusion of nanoparticles in biofilms is altered by bacterial cell wall hydrophobicity', *Applied and Environmental Microbiology*, vol. 77, no. 1, pp. 367-8.

Hammerl, A & Klapötke, TM 2006, 'Nitrogen: Inorganic Chemistry', *Encyclopedia of Inorganic Chemistry*.

Harms, L & Owen, C 2004, 'Keys for controlling nitrification', in *American Water Works Association Water Quality Technology Conference*.

Harper, S, Mozdzanowski, J & Speicher, D 1998, 'Two-dimensional gel electrophoresis', *Current protocols in protein science*, vol. 11, no. 1, pp. 10.4. 1-.4. 36.

Harper, SC, Manoharan, R, Mavinic, DS & Randall, CW 1996, 'Chromium and nickel toxicity during the biotreatment of high ammonia landfill leachate', *Water environment research*, vol. 68, no. 1, pp. 19-24.

Harrington, GW, Noguera, DR, Kandou, AI & Vanhoven, DJ 2002, 'Pilot-scale evaluation of nitrification control strategies', *Journal (American Water Works Association)*, vol. 94, no. 11, pp. 78-89.

Haurowitz, F & Koshland, DE 2017, *protein*, Encyclopædia Britannica, inc. , viewed November 07, 2017 2017, <https://www.britannica.com/science/protein >.

Herath, BS 2014, 'Microbial factors accelerating chloramine decay in chloraminated systems'.

Herath, BS, Sathasivan, A & Lam, HI 2015, 'Can microbes significantly accelerate chloramine decay without severe nitrification?', *International biodeterioration & biodegradation*, vol. 102, pp. 231-6.

Herath, BS, Torres, A & Sathasivan, A 2018, 'Effects of feed water NOM variation on chloramine demand from chloramine-decaying soluble microbial products during rechloramination', *Chemosphere*, vol. 212, pp. 744-54.

Hijnen, W, Schurer, R, Martijn, B, Bahlman, J, Hoogenboezem, W & van der Wielen, P 2014, 'Removal of easily and more complex biodegradable NOM by full-scale BAC filters to produce biological stable drinking water', in *Progress in Slow Sand and Alternative Biofiltration Processes*, IWA publishing London, UK, pp. 413-21.

Hockenbury, MR, Grady, C & Daigger, GT 1977, 'Factors affecting nitrification', *Journal of the Environmental Engineering Division*, vol. 103, no. 1, pp. 9-19.

Hoefel, D 2011, 'Flow Cytometry and PCR Approaches to Investigate Chloramination Instability-First detection of ammonia-oxidising Archaea in a chloraminated system', *Water-Australian Water and Wastewater Association*, vol. 38, no. 4, p. 75.

Hoefel, D, Monis, PT, Grooby, WL, Andrews, S & Saint, CP 2005, 'Culture-independent techniques for rapid detection of bacteria associated with loss of chloramine residual in a drinking water system', *Applied and Environmental Microbiology*, vol. 71, no. 11, pp. 6479-88.

Hoigné, J & Bader, H 1988, 'The formation of trichloronitromethane (chloropicrin) and chloroform in a combined ozonation/chlorination treatment of drinking water', *water research*, vol. 22, no. 3, pp. 313-9.

Holinger, EP, Ross, KA, Robertson, CE, Stevens, MJ, Harris, JK & Pace, NR 2014, 'Molecular analysis of point-of-use municipal drinking water microbiology', *water research*, vol. 49, pp. 225-35.

Hooper, AB, Terry, KR & Maxwell, PC 1977, 'Hydroxylamine oxidoreductase of Nitrosomonas. Oxidation of diethyldithiocarbamate concomitant with stimulation of nitrite synthesis', *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, vol. 462, no. 1, pp. 141-52.

Hu, A, Yao, T, Jiao, N, Liu, Y, Yang, Z & Liu, X 2010, 'Community structures of ammonia-oxidising archaea and bacteria in high-altitude lakes on the Tibetan Plateau', *Freshwater Biology*, vol. 55, no. 11, pp. 2375-90.

Hua, G & Reckhow, DA 2008, 'DBP formation during chlorination and chloramination: effect of reaction time, pH, dosage, and temperature', *Journal-American Water Works Association*, vol. 100, no. 8, pp. 82-95.

Hwang, C, Ling, F, Andersen, GL, LeChevallier, MW & Liu, W-T 2012, 'Microbial community dynamics of an urban drinking water distribution system subjected to phases of chloramination and chlorination treatments', *Applied and Environmental Microbiology*, pp. AEM. 01892-12.

Ibrahim, AM 2018, '38 HEAVY METAL INHIBITION OF RESTING NITRIFYING BACTERIA', in *Proceedings of the 43rd Industrial Waste Conference May 1988, Purdue University*, p. 38.

Ike, N, Wolfe, R & Means, E 1988, 'Nitrifying bacteria in a chloraminated drinking water system', *Water Science and Technology*, vol. 20, no. 11-12, pp. 441-4.

Jafvert, CT & Valentine, RL 1992, 'Reaction scheme for the chlorination of ammoniacal water', *Environmental science & technology*, vol. 26, no. 3, pp. 577-86.

Janga, N, Ren, X, Kim, G, Ahn, C, Cho, J & Kim, IS 2007, 'Characteristics of soluble microbial products and extracellular polymeric substances in the membrane bioreactor for water reuse', *Desalination*, vol. 202, no. 1-3, pp. 90-8.

Jarusutthirak, C & Amy, G 2007, 'Understanding soluble microbial products (SMP) as a component of effluent organic matter (EfOM)', *water research*, vol. 41, no. 12, pp. 2787-93.

Jarvis, P, Mergen, M, Banks, J, McIntosh, B, Parsons, SA & Jefferson, B 2008, 'Pilot scale comparison of enhanced coagulation with magnetic resin plus coagulation systems', *Environmental science & technology*, vol. 42, no. 4, pp. 1276-82.

Jiang, T, Myngheer, S, De Pauw, DJ, Spanjers, H, Nopens, I, Kennedy, MD, Amy, G & Vanrolleghem, PA 2008, 'Modelling the production and degradation of soluble microbial products (SMP) in membrane bioreactors (MBR)', *water research*, vol. 42, no. 20, pp. 4955-64.

Kabsch-Korbutowicz, M 2005, 'Effect of Al coagulant type on natural organic matter removal efficiency in coagulation/ultrafiltration process', *Desalination*, vol. 185, no. 1-3, pp. 327-33.

Karanfil, T, Kitis, M, Kilduff, JE & Wigton, A 1999, 'Role of granular activated carbon surface chemistry on the adsorption of organic compounds. 2. Natural organic matter', *Environmental science & technology*, vol. 33, no. 18, pp. 3225-33.

Karim, M & LeChevallier, M 2006, 'Alternative treatments for nitrification control in chloraminating utilities', in *Proc. 2006 AWWA Ann. Conf., San Antonio*.

Karnik, B, Davies, S, Baumann, M & Masten, S 2005, 'The effects of combined ozonation and filtration on disinfection by-product formation', *water research*, vol. 39, no. 13, pp. 2839-50.

Kim, D-J, Lee, D-I & Keller, J 2006, 'Effect of temperature and free ammonia on nitrification and nitrite accumulation in landfill leachate and analysis of its nitrifying bacterial community by FISH', *Bioresource technology*, vol. 97, no. 3, pp. 459-68.

Kim, H-C & Yu, M-J 2005, 'Characterization of natural organic matter in conventional water treatment processes for selection of treatment processes focused on DBPs control', *water research*, vol. 39, no. 19, pp. 4779-89.

Kim, J, Cai, Z & Benjamin, MM 2008, 'Effects of adsorbents on membrane fouling by natural organic matter', *Journal of membrane science*, vol. 310, no. 1-2, pp. 356-64.

Kim, J, Cho, M, Oh, B, Choi, S & Yoon, J 2004, 'Control of bacterial growth in water using synthesized inorganic disinfectant', *Chemosphere*, vol. 55, no. 5, pp. 775-80.

Kim, K-Y, Kim, H-S, Kim, J, Nam, J-W, Kim, J-M & Son, S 2009, 'A hybrid microfiltration–granular activated carbon system for water purification and wastewater reclamation/reuse', *Desalination*, vol. 243, no. 1-3, pp. 132-44.

Kindaichi, T, Ito, T & Okabe, S 2004, 'Ecophysiological interaction between nitrifying bacteria and heterotrophic bacteria in autotrophic nitrifying biofilms as determined by microautoradiography-fluorescence in situ hybridization', *Applied and Environmental Microbiology*, vol. 70, no. 3, pp. 1641-50.

Kingston, HM & Haswell, SJ 1997, *Microwave-enhanced chemistry*, American Chemical Society.

Kirmeyer, GJ 2004, *Optimizing chloramine treatment*, American Water Works Association.

Kishinevsky, B, Sen, D & Weaver, R 1992, 'Effect of high root temperature on Bradyrhizobium-peanut symbiosis', *Plant and soil*, vol. 143, no. 2, pp. 275-82.

Klueh, U, Wagner, V, Kelly, S, Johnson, A & Bryers, J 2000, 'Efficacy of silver-coated fabric to prevent bacterial colonization and subsequent device-based biofilm formation', *Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, vol. 53, no. 6, pp. 621-31.

Klymenko, NA, Kozyatnyk, IP & Savchyna, LA 2010, 'Removing of fulvic acids by ozonation and biological active carbon filtration', *water research*, vol. 44, no. 18, pp. 5316-22.

Könneke, M, Bernhard, AE, José, R, Walker, CB, Waterbury, JB & Stahl, DA 2005, 'Isolation of an autotrophic ammonia-oxidizing marine archaeon', *Nature*, vol. 437, no. 7058, pp. 543-6.

Korotta-Gamage, SM & Sathasivan, A 2017, 'A review: Potential and challenges of biologically activated carbon to remove natural organic matter in drinking water purification process', *Chemosphere*, vol. 167, pp. 120-38.

Korshin, G, Chow, CW, Fabris, R & Drikas, M 2009, 'Absorbance spectroscopy-based examination of effects of coagulation on the reactivity of fractions of natural organic matter with varying apparent molecular weights', *water research*, vol. 43, no. 6, pp. 1541-8.

Koskinen, R, Ali-Vehmas, T, Kämpfer, P, Laurikkala, M, Tsitko, I, Kostyal, E, Atroshi, F & Salkinoja-Salonen, M 2000, 'Characterization of Sphingomonas isolates from Finnish and

Swedish drinking water distribution systems', *Journal of applied microbiology*, vol. 89, no. 4, pp. 687-96.

Krishna, KB 2012, 'Understanding Microbial Chloramine Decay Mechanism in Water Distribution System', PhD thesis, Curtin University.

Krishna, KB & Sathasivan, A 2010, 'Does an unknown mechanism accelerate chemical chloramine decay in nitrifying waters?', *Journal American Water Works Association*, vol. 102, no. 10, pp. 82-90.

Krishna, KB, Sathasivan, A & Garbin, S 2013, 'Wider presence of accelerated chemical chloramine decay in severely nitrifying conditions', *Water Science and Technology: Water Supply*, vol. 13, no. 4, pp. 1090-8.

Krishna, KB, Sathasivan, A & Ginige, MP 2013, 'Microbial community changes with decaying chloramine residuals in a lab-scale system', *water research*, vol. 47, no. 13, pp. 4666-79.

Krishna, KB, Sathasivan, A & Kastl, G 2014, 'Effectiveness of breakpoint chlorination to reduce accelerated chemical chloramine decay in severely nitrified bulk waters', *Chemosphere*, vol. 117, pp. 692-700.

Krishna, KB, Sathasivan, A & Sarker, DC 2012, 'Evidence of soluble microbial products accelerating chloramine decay in nitrifying bulk water samples', *water research*, vol. 46, no. 13, pp. 3977-88.

Kristiana, I, Joll, C & Heitz, A 2011, 'Powdered activated carbon coupled with enhanced coagulation for natural organic matter removal and disinfection by-product control: Application in a Western Australian water treatment plant', *Chemosphere*, vol. 83, no. 5, pp. 661-7.

Kronman, MJ, Holmes, LG & Robbins, FM 1971, 'Inter-and intramolecular interactions of α-lactalbumin X. Effect of acylation of tyrosyl and lysyl side chains on molecular conformations', *Journal of Biological Chemistry*, vol. 246, no. 6, pp. 1909-21.

Krümmel, A & Harms, H 1982, 'Effect of organic matter on growth and cell yield of ammonia-oxidizing bacteria', *Archives of Microbiology*, vol. 133, no. 1, pp. 50-4.

Lalley, J, Dionysiou, DD, Varma, RS, Shankara, S, Yang, DJ & Nadagouda, MN 2014, 'Silver-based antibacterial surfaces for drinking water disinfection—an overview', *Current Opinion in Chemical Engineering*, vol. 3, pp. 25-9.

Laspidou, CS & Rittmann, BE 2002, 'A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass', *water research*, vol. 36, no. 11, pp. 2711-20.

Lautenschlager, K, Boon, N, Wang, Y, Egli, T & Hammes, F 2010, 'Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition', *water research*, vol. 44, no. 17, pp. 4868-77.

Lautenschlager, K, Hwang, C, Liu, W-T, Boon, N, Köster, O, Vrouwenvelder, H, Egli, T & Hammes, F 2013, 'A microbiology-based multi-parametric approach towards assessing biological stability in drinking water distribution networks', *water research*, vol. 47, no. 9, pp. 3015-25.

Le Dantec, C, Duguet, J-P, Montiel, A, Dumoutier, N, Dubrou, S & Vincent, V 2002, 'Chlorine disinfection of atypical mycobacteria isolated from a water distribution system', *Applied and Environmental Microbiology*, vol. 68, no. 3, pp. 1025-32.

Lee, CO, Boe-Hansen, R, Musovic, S, Smets, B, Albrechtsen, H-J & Binning, P 2014, 'Effects of dynamic operating conditions on nitrification in biological rapid sand filters for drinking water treatment', *water research*, vol. 64, pp. 226-36.

Lee, DY & Rhee, GY 1997, 'KINETICS OF CELL DEATH IN THE CYANOBACTERIUM ANABAENA FLOS-AQUAE AND THE PRODUCTION OF DISSOLVED ORGANIC CARBON 1', *Journal of Phycology*, vol. 33, no. 6, pp. 991-8.

Lehrach, H, Diamond, D, Wozney, JM & Boedtker, H 1977, 'RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination', *Biochemistry*, vol. 16, no. 21, pp. 4743-51.

Lenntech, K 2004, 'Water treatment and air purification', *Published by Rotter Dam Seweg, Netherlands*.

Lew, A, Krutzik, PO, Hart, ME & Chamberlin, AR 2002, 'Increasing rates of reaction: microwave-assisted organic synthesis for combinatorial chemistry', *Journal of combinatorial chemistry*, vol. 4, no. 2, pp. 95-105.

Lewandowski, Z & Beyenal, H 2013, *Fundamentals of biofilm research*, CRC press.

Li, F, Yuasa, A, Ebie, K, Azuma, Y, Hagishita, T & Matsui, Y 2002, 'Factors affecting the adsorption capacity of dissolved organic matter onto activated carbon: modified isotherm analysis', *water research*, vol. 36, no. 18, pp. 4592-604.

Li, Q, Mahendra, S, Lyon, DY, Brunet, L, Liga, MV, Li, D & Alvarez, PJ 2008, 'Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications', *water research*, vol. 42, no. 18, pp. 4591-602.

Li, X, Wang, H, Zhang, Y, Hu, C & Yang, M 2014, 'Characterization of the bacterial communities and iron corrosion scales in drinking groundwater distribution systems with chlorine/chloramine', *International biodeterioration & biodegradation*, vol. 96, pp. 71-9.

Liau, S, Read, D, Pugh, W, Furr, J & Russell, A 1997, 'Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterialaction of silver ions', *Letters in applied microbiology*, vol. 25, no. 4, pp. 279-83.

Lieu, NI, Wolfe, RL & Means, EG 1993, 'Optimizing chloramine disinfection for the control of nitrification', *Journal of the American Water Works Association*, vol. 85, no. 2, pp. 84-90.

Lipponen, MT, Martikainen, PJ, Vasara, RE, Servomaa, K, Zacheus, O & Kontro, MH 2004, 'Occurrence of nitrifiers and diversity of ammonia-oxidizing bacteria in developing drinking water biofilms', *water research*, vol. 38, no. 20, pp. 4424-34.

Lipponen, MT, Suutari, MH & Martikainen, PJ 2002, 'Occurrence of nitrifying bacteria and nitrification in Finnish drinking water distribution systems', *water research*, vol. 36, no. 17, pp. 4319-29.

Liu, H, Cheng, F & Wang, D 2009, 'Interaction of ozone and organic matter in coagulation with inorganic polymer flocculant-PACl: Role of organic components', *Desalination*, vol. 249, no. 2, pp. 596-601.

Liu, H, Wang, D, Wang, M, Tang, H & Yang, M 2007, 'Effect of pre-ozonation on coagulation with IPF–PACls: Role of coagulant speciation', *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 294, no. 1-3, pp. 111-6.

Liu, J, Ren, H, Ye, X, Wang, W, Liu, Y, Lou, L, Cheng, D, He, X, Zhou, X & Qiu, S 2017, 'Bacterial community radial-spatial distribution in biofilms along pipe wall in chlorinated drinking water distribution system of East China', *Applied microbiology and biotechnology*, vol. 101, no. 2, pp. 749-59.

Liu, R, Yu, Z, Zhang, H, Yang, M, Shi, B & Liu, X 2012, 'Diversity of bacteria and mycobacteria in biofilms of two urban drinking water distribution systems', *Canadian journal of microbiology*, vol. 58, no. 3, pp. 261-70.

Liu, X, Wang, J, Liu, T, Kong, W, He, X, Jin, Y & Zhang, B 2015, 'Effects of Assimilable Organic Carbon and Free Chlorine on Bacterial Growth in Drinking Water', *PloS one*, vol. 10, no. 6, p. e0128825.

Liu, Y-Q, Liu, Y & Tay, J-H 2004, 'The effects of extracellular polymeric substances on the formation and stability of biogranules', *Applied microbiology and biotechnology*, vol. 65, no. 2, pp. 143-8.

Liu, Y & Fang, HH 2003, 'Influences of extracellular polymeric substances (EPS) on flocculation, settling, and dewatering of activated sludge'.

Liu, Y, Sun, J, Peng, L, Wang, D, Dai, X & Ni, B-J 2016, 'Assessment of heterotrophic growth supported by soluble microbial products in anammox biofilm using multidimensional modeling', *Scientific reports*, vol. 6, p. 27576.

Liu, Z-P, Wang, B-J, Liu, Y-H & Liu, S-J 2005, 'Novosphingobium taihuense sp. nov., a novel aromatic-compound-degrading bacterium isolated from Taihu Lake, China', *International journal of systematic and evolutionary microbiology*, vol. 55, no. 3, pp. 1229-32.

Lu, J, Zhang, T, Ma, J & Chen, Z 2009, 'Evaluation of disinfection by-products formation during chlorination and chloramination of dissolved natural organic matter fractions isolated from a filtered river water', *Journal of Hazardous Materials*, vol. 162, no. 1, pp. 140-5.

Macnab, RM 1999, 'The bacterial flagellum: reversible rotary propellor and type III export apparatus', *Journal of bacteriology*, vol. 181, no. 23, pp. 7149-53.

Macqueen, DJ & Gubry-Rangin, C 2016, 'Molecular adaptation of ammonia monooxygenase during independent pH specialization in Thaumarchaeota', *Molecular ecology*, vol. 25, no. 9, pp. 1986-99.

Magnusson, U, Salopek-Sondi, B, Luck, LA & Mowbray, SL 2004, 'X-ray structures of the leucine-binding protein illustrate conformational changes and the basis of ligand specificity', *Journal of Biological Chemistry*, vol. 279, no. 10, pp. 8747-52.

Manz, W, Kalmbach, S & Szewzyk, U 2015, 'Incertae Sedis I. Aquabacterium', *Bergey's Manual of Systematics of Archaea and Bacteria*, pp. 1-6.

Margerum, DW, Schurter, LM, Hobson, J & Moore, EE 1994, 'Water chlorination chemistry: nonmetal redox kinetics of chloramine and nitrite ion', *Environmental science & technology*, vol. 28, no. 2, pp. 331-7.

Martin, G & Richard, Y 1982, 'Elements of the inhibition of nitrifying bacteria', *Water Science and Technology*, vol. 14, no. 6-7, p. 781.

Martiny, AC, Jørgensen, TM, Albrechtsen, H-J, Arvin, E & Molin, S 2003, 'Long-term succession of structure and diversity of a biofilm formed in a model drinking water distribution system', *Applied and Environmental Microbiology*, vol. 69, no. 11, pp. 6899-907.

Matilainen, A, Vepsäläinen, M & Sillanpää, M 2010, 'Natural organic matter removal by coagulation during drinking water treatment: a review', *Advances in colloid and interface science*, vol. 159, no. 2, pp. 189-97.

McGuire, MJ, Lieu, NI & Pearthree, MS 1999, 'Using the chlorite ion to control nitrification', *American Water Works Association. Journal*, vol. 91, no. 10, p. 52.

McGuire, MJ, Pearthree, MS, Blute, NK, Arnold, KF & Hoogerwerf, T 2006, 'Nitrification control by chlorite ion at pilot scale', *Journal‑American Water Works Association*, vol. 98, no. 1, pp. 95-105.

Mergen, MR, Jefferson, B, Parsons, SA & Jarvis, P 2008, 'Magnetic ion-exchange resin treatment: Impact of water type and resin use', *water research*, vol. 42, no. 8-9, pp. 1977-88.

Metcalfe, D, Rockey, C, Jefferson, B, Judd, S & Jarvis, P 2015, 'Removal of disinfection by-product precursors by coagulation and an innovative suspended ion exchange process', *water research*, vol. 87, pp. 20-8.

Mir, J, Morato, J & Ribas, F 1997, 'Resistance to chlorine of freshwater bacterial strains', *Journal of applied microbiology*, vol. 82, no. 1, pp. 7-18.

Molmeret, M, Horn, M, Wagner, M, Santic, M & Kwaik, YA 2005, 'Amoebae as training grounds for intracellular bacterial pathogens', *Applied and Environmental Microbiology*, vol. 71, no. 1, pp. 20-8.

Molnar, J, Agbaba, J, Dalmacija, B, Tubić, A, Krčmar, D, Maletić, S & Tomašević, D 2013, 'The effects of matrices and ozone dose on changes in the characteristics of natural organic matter', *Chemical Engineering Journal*, vol. 222, pp. 435-43.

Moradi, S, Liu, S, Chow, CW, van Leeuwen, J, Cook, D, Drikas, M & Amal, R 2017, 'Developing a chloramine decay index to understand nitrification: A case study of two chloraminated drinking water distribution systems', *Journal of Environmental Sciences*, vol. 57, pp. 170-9.

More, T, Yadav, JSS, Yan, S, Tyagi, RD & Surampalli, RY 2014, 'Extracellular polymeric substances of bacteria and their potential environmental applications', *Journal of environmental management*, vol. 144, pp. 1-25.

Moreno-Castilla, C 2004, 'Adsorption of organic molecules from aqueous solutions on carbon materials', *Carbon*, vol. 42, no. 1, pp. 83-94.

Morones, JR, Elechiguerra, JL, Camacho, A, Holt, K, Kouri, JB, Ramírez, JT & Yacaman, MJ 2005, 'The bactericidal effect of silver nanoparticles', *Nanotechnology*, vol. 16, no. 10, p. 2346.

Morran, J, Drikas, M, Cook, D & Bursill, D 2004, 'Comparison of MIEX® treatment and coagulation on NOM character', *Water Science and Technology: Water Supply*, vol. 4, no. 4, pp. 129-37.

Murray, CA & Parsons, SA 2004, 'Removal of NOM from drinking water: Fenton's and photo-Fenton's processes', *Chemosphere*, vol. 54, no. 7, pp. 1017-23.

Nagymáté, Z, Homonnay, ZG & Márialigeti, K 2016, 'Investigation of Archaeal and Bacterial community structure of five different small drinking water networks with special regard to the nitrifying microorganisms', *Microbiological research*, vol. 188, pp. 80-9.

Narberhaus, F, Weiglhofer, W, Fischer, H-M & Hennecke, H 1996, 'The Bradyrhizobium japonicum rpoH1 gene encoding a sigma 32-like protein is part of a unique heat shock gene cluster together with groESL1 and three small heat shock genes', *Journal of bacteriology*, vol. 178, no. 18, pp. 5337-46.

Neden, DG, Jones, RJ, Smith, JR, Kirmeyer, GJ & Foust, GW 1992, 'Comparing chlorination and chloramination for controlling bacterial regrowth', *Journal‐American Water Works Association*, vol. 84, no. 7, pp. 80-8.

Nel, A 2005, 'Air pollution-related illness: effects of particles', *Science*, vol. 308, no. 5723, pp. 804-6.

Neu, TR, Flemming, H-C & Wingender, J 1999, *Microbial extracellular polymeric substances: Characterization, structure and function*, Springer.

Newcombe, G 1999, 'Charge vs. porosity—some influences on the adsorption of natural organic matter (NOM) by activated carbon', *Water Science and Technology*, vol. 40, no. 9, pp. 191-8.

Newcombe, G & Drikas, M 1997, 'Adsorption of NOM onto activated carbon: electrostatic and non-electrostatic effects', *Carbon*, vol. 35, no. 9, pp. 1239-50.

Noguera, D, Goel, R, Harrington, G & Yilmaz, L 2009, *Identification of Heterotrophic Bacteria that Colonize Chloraminated Crinking Water Distribution Systems*, Water Environment Research Foundation.

Norton, CD & LeChevallier, MW 1997, 'Chloramination: its effect on distribution system water quality', *Journal‐American Water Works Association*, vol. 89, no. 7, pp. 66-77.

Nowlin, M, Summers, RS & Work, L 2001, 'Chlorine and chloramine decay in drinking water: the impact of temperature', University of Colorado.

Nuengjamnong, C, Kweon, JH, Cho, J, Polprasert, C & Ahn, K-H 2005, 'Membrane fouling caused by extracellular polymeric substances during microfiltration processes', *Desalination*, vol. 179, no. 1-3, pp. 117-24.

Ødegaard, H, Østerhus, S, Melin, E & Eikebrokk, B 2010, 'NOM removal technologies–Norwegian experiences', *Drinking Water Engineering and Science*, vol. 3, no. 1, pp. 1-9.

Odell, LH, Kirmeyer, GJ, Wilczak, A & Jacangelo, JG 1996, 'Controlling nitrification in chloraminated systems', *American Water Works Association. Journal*, vol. 88, no. 7, p. 86.

Ohashi, A, de Silva, DV, Mobarry, B, Manem, JA, Stahl, DA & Rittmann, BE 1995, 'Influence of substrate C/N ratio on the structure of multi-species biofilms consisting of nitrifiers and heterotrophs', *Water Science and Technology*, vol. 32, no. 8, pp. 75-84.

Okabe, S, Kindaichi, T & Ito, T 2005, 'Fate of 14C-labeled microbial products derived from nitrifying bacteria in autotrophic nitrifying biofilms', *Applied and Environmental Microbiology*, vol. 71, no. 7, pp. 3987-94.

Okpara, C, Oparaku, N & Ibeto, C 2011, 'An overview of water disinfection in developing countries and potentials of renewable energy', *J. Environ. Sci. Technol*, vol. 4, no. 1, pp. 18-30.

Oldenburg, PS, Regan, JM, Harrington, GW & Noguera, DR 2002, 'Kinetics of" Nitrosomonas europaea" INACTIVATION by chloramine', *Journal (American Water Works Association)*, vol. 94, no. 10, pp. 100-10.

Otsuki, A & Hanya, T 1972, 'Production of Dissolved Organic Matter from Dead Green Algal Cells. II. Anaerobic Microbial DECOMPOSITION1', *Limnology and Oceanography*, vol. 17, no. 2, pp. 258-64.

Painter, HA 2013, 'Microbial transformations of inorganic nitrogen', in *Proceedings of the Conference on Nitrogen As a Water Pollutant*, pp. 3-29.

Pan, P & Umbreit, W 1972, 'Growth of mixed cultures of autotrophic and heterotrophic organisms', *Canadian journal of microbiology*, vol. 18, no. 2, pp. 153-6.

Park, H-D, Wells, GF, Bae, H, Criddle, CS & Francis, CA 2006, 'Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors', *Applied and Environmental Microbiology*, vol. 72, no. 8, pp. 5643-7.

Parkin, GF & McCarty, PL 1981, 'A comparison of the characteristics of soluble organic nitrogen in untreated and activated sludge treated wastewaters', *water research*, vol. 15, no. 1, pp. 139-49.

Parsons, S, Goslan, E, Jarvis, P, Hurst, A & Fearing, D 2002, 'NOM–the relationship between character and treatability', in *International Conference on Natural Organic Matter Characterisation and Treatment*.

Patel, RN, Hou, CT, Laskin, AI & Felix, A 1982, 'Microbial oxidation of hydrocarbons: properties of a soluble methane monooxygenase from a facultative methane-utilizing organism, Methylobacterium sp. strain CRL-26', *Applied and Environmental Microbiology*, vol. 44, no. 5, pp. 1130-7.

Pedahzur, R, Shuval, H & Ulitzur, S 1997, 'Silver and hydrogen peroxide as potential drinking water disinfectants: their bactericidal effects and possible modes of action', *Water Science and Technology*, vol. 35, no. 11-12, pp. 87-93.

Pelletier, P & Stottmeier, K 1988, 'Mycobacteria in public water supplies: comparative resistance to chlorine', *Microbiological sciences*, vol. 5, no. 5, pp. 147-8.

Pernthaler, J, Glöckner, F-O, Unterholzner, S, Alfreider, A, Psenner, R & Amann, R 1998, 'Seasonal community and population dynamics of pelagic bacteria and archaea in a high mountain lake', *Applied and Environmental Microbiology*, vol. 64, no. 11, pp. 4299-306.

Pinto, AJ, Marcus, DN, Ijaz, UZ, Bautista-de lose Santos, QM, Dick, GJ & Raskin, L 2016, 'Metagenomic evidence for the presence of comammox nitrospira-like bacteria in a drinking water system', *Msphere*, vol. 1, no. 1, pp. e00054-15.

Pinto, AJ, Schroeder, J, Lunn, M, Sloan, W & Raskin, L 2014, 'Spatial-temporal survey and occupancy-abundance modeling to predict bacterial community dynamics in the drinking water microbiome', *MBio*, vol. 5, no. 3, pp. e01135-14.

Pressman, JG, Lee, WH, Bishop, PL & Wahman, DG 2012, 'Effect of free ammonia concentration on monochloramine penetration within a nitrifying biofilm and its effect on activity, viability, and recovery', *water research*, vol. 46, no. 3, pp. 882-94.

Qin, W, Amin, SA, Lundeen, RA, Heal, KR, Martens-Habbena, W, Turkarslan, S, Urakawa, H, Costa, KC, Hendrickson, EL & Wang, T 2017, 'Stress response of a marine ammonia-oxidizing archaeon informs physiological status of environmental populations', *The ISME journal*, vol. 12, no. 2, p. 508.

Quinlivan, PA, Li, L & Knappe, DR 2005, 'Effects of activated carbon characteristics on the simultaneous adsorption of aqueous organic micropollutants and natural organic matter', *water research*, vol. 39, no. 8, pp. 1663-73.

Rahman, MS, Encarnacion, G & Camper, AK 2011, 'Nitrification and potential control mechanisms in simulated premises plumbing', *water research*, vol. 45, no. 17, pp. 5511-22.

Ramalingam, B, Sekar, R, Boxall, J & Biggs, C 2013, 'Aggregation and biofilm formation of bacteria isolated from domestic drinking water', *Water Science and Technology: Water Supply*, vol. 13, no. 4, pp. 1016-23.

Rämö, J & Sillanpää, M 2001, 'Degradation of EDTA by hydrogen peroxide in alkaline conditions', *Journal of cleaner production*, vol. 9, no. 3, pp. 191-5.

Rand, JL & Gagnon, GA 2008, 'Loss of chlorine, chloramine or chlorine dioxide concentration following exposure to UV light', *Journal of Water Supply: Research and Technology-Aqua*, vol. 57, no. 2, pp. 127-32.

Ratte, HT 1999, 'Bioaccumulation and toxicity of silver compounds: a review', *Environmental Toxicology and Chemistry*, vol. 18, no. 1, pp. 89-108.

Regan, JM 2001, *Microbial ecology of nitrification in chloraminated drinking water distribution systems*, University of Wisconsin--Madison.

Regan, JM, Harrington, GW, Baribeau, H, De Leon, R & Noguera, DR 2003, 'Diversity of nitrifying bacteria in full-scale chloraminated distribution systems', *water research*, vol. 37, no. 1, pp. 197-205.

Regan, JM, Harrington, GW & Noguera, DR 2002, 'Ammonia-and nitrite-oxidizing bacterial communities in a pilot-scale chloraminated drinking water distribution system', *Applied and Environmental Microbiology*, vol. 68, no. 1, pp. 73-81.

Richardson, S, Thruston, A, Caughran, T, Chen, P, Collette, T, Schenck, K, Lykins, B, Rav-Acha, C & Glezer, V 2000, 'Identification of new drinking water disinfection by-products from ozone, chlorine dioxide, chloramine, and chlorine', *Water, Air, and Soil Pollution*, vol. 123, no. 1-4, pp. 95-102.

Ridgway, H & Olson, B 1982, 'Chlorine resistance patterns of bacteria from two drinking water distribution systems', *Applied and Environmental Microbiology*, vol. 44, no. 4, pp. 972-87.

Riess, F, Dörner, U, Schiffler, B & Benz, R 2001, 'Study of the properties of a channel-forming protein of the cell wall of the gram-positive bacterium Mycobacterium phlei', *The Journal of membrane biology*, vol. 182, no. 2, pp. 147-57.

Rio, DC, Ares, M, Hannon, GJ & Nilsen, TW 2010, 'Polyacrylamide gel electrophoresis of RNA', *Cold Spring Harbor Protocols*, vol. 2010, no. 6, p. pdb. prot5444.

Rittmann, BE 1984, 'Achieving biologically stable drinking water', *Journal of the American Water Works Association*, vol. 76, no. 10, pp. 106-14.

Rittmann, BE 2018, 'Biofilms, active substrata, and me', *water research*, vol. 132, pp. 135-45.

Rittmann, BE, Bae, W, Namkung, E & Lu, C-J 1987, 'A critical evaluation of microbial product formation in biological processes', *Water Science and Technology*, vol. 19, no. 3-4, pp. 517-28.

Rittmann, BE & Manem, JA 1992, 'Development and experimental evaluation of a steady-state, multispecies biofilm model', *Biotechnology and bioengineering*, vol. 39, no. 9, pp. 914-22.

Rittmann, BE, Regan, JM & Stahl, DA 1994, 'Nitrification as a source of soluble organic substrate in biological treatment', *Water Science and Technology*, vol. 30, no. 6, p. 1.

Rook, JJ 1974, 'Formation of haloforms during chlorination of natural waters', *Water Treat. Exam.*, vol. 23, pp. 234-43.

Russell, A & Hugo, W 1994, '7 antimicrobial activity and action of silver', in *Progress in medicinal chemistry*, Elsevier, vol. 31, pp. 351-70.

Saby, S, Leroy, P & Block, J-C 1999, 'Escherichia coli resistance to chlorine and glutathione synthesis in response to oxygenation and starvation', *Applied and Environmental Microbiology*, vol. 65, no. 12, pp. 5600-3.

Sambhy, V, MacBride, MM, Peterson, BR & Sen, A 2006, 'Silver bromide nanoparticle/polymer composites: dual action tunable antimicrobial materials', *Journal of the American Chemical Society*, vol. 128, no. 30, pp. 9798-808.

Sarker, DC 2012, 'Modelling inhibition of microbes responsible for acceleration of chloramine decay in water supply system'.

Sarker, DC, Sathasivan, A, Joll, CA & Heitz, A 2013, 'Modelling temperature effects on ammonia-oxidising bacterial biostability in chloraminated systems', *Science of The Total Environment*, vol. 454, pp. 88-98.

Sarker, DC, Sathasivan, A & Rittmann, BE 2015, 'Modelling combined effect of chloramine and copper on ammonia-oxidizing microbial activity using a biostability approach', *water research*, vol. 84, pp. 190-7.

Sartory, DP 2004, 'Heterotrophic plate count monitoring of treated drinking water in the UK: a useful operational tool', *International journal of food microbiology*, vol. 92, no. 3, pp. 297-306.

Sathasivan, A & Bal Krishna, K 2012, 'Major mechanism (s) of chloramine decay in rechloraminated laboratory scale system waters', *Desalination and Water Treatment*, vol. 47, no. 1-3, pp. 112-9.

Sathasivan, A, Fisher, I & Kastl, G 2005, 'Simple method for quantifying microbiologically assisted chloramine decay in drinking water', *Environmental science & technology*, vol. 39, no. 14, pp. 5407-13.

Sathasivan, A, Fisher, I & Kastl, G 2010, 'Application of the microbial decay factor to maintain chloramine in large tanks', *Journal-American Water Works Association*, vol. 102, no. 4, pp. 94-103.

Sathasivan, A, Fisher, I & Tam, T 2008, 'Onset of severe nitrification in mildly nitrifying chloraminated bulk waters and its relation to biostability', *water research*, vol. 42, no. 14, pp. 3623-32.

Sawade, E, Monis, P, Cook, D & Drikas, M 2016, 'Is nitrification the only cause of microbiologically induced chloramine decay?', *water research*, vol. 88, pp. 904-11.

Schleper, C & Nicol, GW 2010, 'Ammonia-oxidising archaea–physiology, ecology and evolution', in *Advances in microbial physiology*, Elsevier, vol. 57, pp. 1-41.

Schwering, M, Song, J, Louie, M, Turner, RJ & Ceri, H 2013, 'Multi-species biofilms defined from drinking water microorganisms provide increased protection against chlorine disinfection', *Biofouling*, vol. 29, no. 8, pp. 917-28.

Seidel, CJ, McGuire, MJ, Summers, RS & Via, S 2005, 'Have utilities switched to chloramines?', *Journal (American Water Works Association)*, vol. 97, no. 10, pp. 87-97.

Sepehri, A & Sarrafzadeh, M-H 2018, 'Effect of nitrifiers community on fouling mitigation and nitrification efficiency in a membrane bioreactor', *Chemical Engineering and Processing-Process Intensification*, vol. 128, pp. 10-8.

Seredyńska-Sobecka, B, Tomaszewska, M & Morawski, AW 2005, 'Removal of micropollutants from water by ozonation/biofiltration process', *Desalination*, vol. 182, no. 1-3, pp. 151-7.

Shah, AD & Mitch, WA 2011, 'Halonitroalkanes, halonitriles, haloamides, and N-nitrosamines: a critical review of nitrogenous disinfection byproduct formation pathways', *Environmental science & technology*, vol. 46, no. 1, pp. 119-31.

Shammas, NK 1986, 'Interactions of temperature, pH, and biomass on the nitrification process', *Journal (Water Pollution Control Federation)*, pp. 52-9.

Sharma, B & Ahlert, R 1977, 'Nitrification and nitrogen removal', *water research*, vol. 11, no. 10, pp. 897-925.

Sheng, G-P, Yu, H-Q & Li, X-Y 2010, 'Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review', *Biotechnology advances*, vol. 28, no. 6, pp. 882-94.

Shi, P, Jia, S, Zhang, X-X, Zhang, T, Cheng, S & Li, A 2013, 'Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water', *water research*, vol. 47, no. 1, pp. 111-20.

Shi, Y, Huang, J, Zeng, G, Gu, Y, Hu, Y, Tang, B, Zhou, J, Yang, Y & Shi, L 2018, 'Evaluation of soluble microbial products (SMP) on membrane fouling in membrane bioreactors (MBRs) at the fractional and overall level: a review', *Reviews in Environmental Science and Bio/Technology*, vol. 17, no. 1, pp. 71-85.

Sicairos-Ruelas, EE, Gerba, CP & Bright, KR 2019, 'Efficacy of copper and silver as residual disinfectants in drinking water', *Journal of Environmental Science and Health, Part A*, vol. 54, no. 2, pp. 146-55.

Sillanpää, M & Matilainen, A 2014, 'NOM removal by coagulation', *Natural organic matter in water: characterization and treatment methods*, vol. 55.

Sillanpää, M, Pirkanniemi, K & Sorokin, A 2009, 'Oxidation of EDTA with H2O2 catalysed by metallophthalocyanines', *Environmental technology*, vol. 30, no. 14, pp. 1593-600.

Silver, S 2003, 'Bacterial silver resistance: molecular biology and uses and misuses of silver compounds', *FEMS microbiology reviews*, vol. 27, no. 2-3, pp. 341-53.

Silvestry-Rodriguez, N, Bright, KR, Slack, DC, Uhlmann, DR & Gerba, CP 2008, 'Silver as a residual disinfectant to prevent biofilm formation in water distribution systems', *Applied and Environmental Microbiology*, vol. 74, no. 5, pp. 1639-41.

Simões, LC, Simões, M, Oliveira, R & Vieira, MJ 2007, 'Potential of the adhesion of bacteria isolated from drinking water to materials', *Journal of basic microbiology*, vol. 47, no. 2, pp. 174-83.

Simoes, LC, Simoes, M & Vieira, MJ 2010, 'Influence of the diversity of bacterial isolates from drinking water on resistance of biofilms to disinfection', *Applied and Environmental Microbiology*, vol. 76, no. 19, pp. 6673-9.

Simões, M, Carvalho, H, Pereira, MO & Vieira, MJ 2003, 'Studies on the behaviour of pseudomonas fluorescens biofilms after ortho-phthalaldehyde treatment', *Biofueling*, vol. 19, no. 3, pp. 151-7.

Simões, M, Pereira, MO & Vieira, MJ 2005, 'Action of a cationic surfactant on the activity and removal of bacterial biofilms formed under different flow regimes', *water research*, vol. 39, no. 2-3, pp. 478-86.

Skadsen, J 1993, 'Nitrification in a distribution system', *Journal (American Water Works Association)*, pp. 95-103.

Slawson, RM, Van Dyke, MI, Lee, H & Trevors, JT 1992, 'Germanium and silver resistance, accumulation, and toxicity in microorganisms', *Plasmid*, vol. 27, no. 1, pp. 72-9.

Sohn, JH, Kwon, KK, Kang, J-H, Jung, H-B & Kim, S-J 2004, 'Novosphingobium pentaromativorans sp. nov., a high-molecular-mass polycyclic aromatic hydrocarbon-degrading bacterium isolated from estuarine sediment', *International journal of systematic and evolutionary microbiology*, vol. 54, no. 5, pp. 1483-7.

Song, Y, Dong, B, Gao, N & Xia, S 2010, 'Huangpu River water treatment by microfiltration with ozone pretreatment', *Desalination*, vol. 250, no. 1, pp. 71-5.

Soto, GE & Hultgren, SJ 1999, 'Bacterial adhesins: common themes and variations in architecture and assembly', *Journal of bacteriology*, vol. 181, no. 4, pp. 1059-71.

Spieck, E, Müller, S, Engel, A, Mandelkow, E, Patel, H & Bock, E 1996, 'Two-dimensional structure of membrane-bound nitrite oxidoreductase from Nitrobacter hamburgensis', *Journal of Structural Biology*, vol. 117, no. 2, pp. 117-23.

Starodub, M & Trevors, J 1989, 'Silver resistance in Escherichia coli R1', *Journal of medical microbiology*, vol. 29, no. 2, pp. 101-10.

Sun, W, Liu, W, Cui, L, Zhang, M & Wang, B 2013, 'Characterization and identification of a chlorine-resistant bacterium, Sphingomonas TS001, from a model drinking water distribution system', *Science of The Total Environment*, vol. 458, pp. 169-75.

Sutherland, IW 2001, 'Biofilm exopolysaccharides: a strong and sticky framework', *Microbiology*, vol. 147, no. 1, pp. 3-9.

Svecevičius, G, Šyvokienė, J, Stasiūnaitė, P & Mickėnienė, L 2005, 'Acute and chronic toxicity of chlorine dioxide (ClO2) and chlorite (ClO2) to rainbow trout (Oncorhynchus mykiss)(4 pp)', *Environmental Science and Pollution Research*, vol. 12, no. 5, pp. 302-5.

Taylor, RH, Falkinham, JO, Norton, CD & LeChevallier, MW 2000, 'Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of Mycobacterium avium', *Applied and Environmental Microbiology*, vol. 66, no. 4, pp. 1702-5.

Thomas, V, Loret, JF, Jousset, M & Greub, G 2008, 'Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant', *Environmental microbiology*, vol. 10, no. 10, pp. 2728-45.

Thurman, RB, Gerba, CP & Bitton, G 1989, 'The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses', *Critical reviews in environmental science and technology*, vol. 18, no. 4, pp. 295-315.

Tijing, LD, Woo, YC, Choi, J-S, Lee, S, Kim, S-H & Shon, HK 2015, 'Fouling and its control in membrane distillation—A review', *Journal of membrane science*, vol. 475, pp. 215-44.

Torvinen, E, Suomalainen, S, Lehtola, MJ, Miettinen, IT, Zacheus, O, Paulin, L, Katila, M-L & Martikainen, PJ 2004, 'Mycobacteria in water and loose deposits of drinking water distribution systems in Finland', *Applied and Environmental Microbiology*, vol. 70, no. 4, pp. 1973-81.

Tran, T, Gray, S, Naughton, R & Bolto, B 2006, 'Polysilicato-iron for improved NOM removal and membrane performance', *Journal of membrane science*, vol. 280, no. 1-2, pp. 560-71.

Tsagkari, E & Sloan, WT 2018, 'Impact of Methylobacterium in the drinking water microbiome on removal of trihalomethanes', *International biodeterioration & biodegradation*.

Tuovinen, OH, Button, KS, Vuorinen, A, Carlson, L, Mair, DM & Yut, LA 1980, 'Bacterial, chemical, and mineralogical characteristics of tubercles in distribution pipelines', *Journal (American Water Works Association)*, pp. 626-35.

Uyak, V, Yavuz, S, Toroz, I, Ozaydin, S & Genceli, EA 2007, 'Disinfection by-products precursors removal by enhanced coagulation and PAC adsorption', *Desalination*, vol. 216, no. 1-3, pp. 334-44.

Uyguner, CS, Suphandag, SA, Kerc, A & Bekbolet, M 2007, 'Evaluation of adsorption and coagulation characteristics of humic acids preceded by alternative advanced oxidation techniques', *Desalination*, vol. 210, no. 1-3, pp. 183-93.

Valentine, R 1985, 'Disappearance of Monochloramine in the Presence of Nitrite. Water Chlorination: Chemistry, Environmental Impact, and Health Effects. Vol. 5 (RL Jolley et al, editors)', *Inc., Chelsea, Mich.*

Valentine, R, Wilber, G, Jolley, R, Condie, L, Johnson, J, Katz, S, Minear, R, Mattice, J & Jacobs, V 1987, *Water Chlorination: Chemistry, Environmental Impact, and Health Effects*, Jolley, RL, Condie, LW, Johnson, JD, Katz, S., Minear, RA, Mattice, JS, Jacobs, VA, Eds.

Valentine, RL 1998, *Chloramine decomposition in distribution system and model waters*, American Water Works Association.

van der Wielen, PW, Voost, S & van der Kooij, D 2009, 'Ammonia-oxidizing bacteria and archaea in groundwater treatment and drinking water distribution systems', *Applied and Environmental Microbiology*, vol. 75, no. 14, pp. 4687-95.

van Kessel, MA, Speth, DR, Albertsen, M, Nielsen, PH, den Camp, HJO, Kartal, B, Jetten, MS & Lücker, S 2015, 'Complete nitrification by a single microorganism', *Nature*, vol. 528, no. 7583, p. 555.

Varin, T, Lovejoy, C, Jungblut, AD, Vincent, WF & Corbeil, J 2012, 'Metagenomic analysis of stress genes in microbial mat communities from Antarctica and the High Arctic', *Appl. Environ. Microbiol.*, vol. 78, no. 2, pp. 549-59.

Velten, S, Boller, M, Köster, O, Helbing, J, Weilenmann, H-U & Hammes, F 2011, 'Development of biomass in a drinking water granular active carbon (GAC) filter', *water research*, vol. 45, no. 19, pp. 6347-54.

Verhagen, FJ & Laanbroek, HJ 1991, 'Competition for ammonium between nitrifying and heterotrophic bacteria in dual energy-limited chemostats', *Applied and Environmental Microbiology*, vol. 57, no. 11, pp. 3255-63.

Verhamme, DT, Prosser, JI & Nicol, GW 2011, 'Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms', *The ISME journal*, vol. 5, no. 6, p. 1067.

Vikesland, PJ, Ozekin, K & Valentine, RL 1998, 'Effect of natural organic matter on monochloramine decomposition: pathway elucidation through the use of mass and redox balances', *Environmental science & technology*, vol. 32, no. 10, pp. 1409-16.

Vikesland, PJ, Ozekin, K & Valentine, RL 2001, 'Monochloramine decay in model and distribution system waters', *water research*, vol. 35, no. 7, pp. 1766-76.

Vikesland, PJ & Valentine, RL 2000, 'Reaction pathways involved in the reduction of monochloramine by ferrous iron', *Environmental science & technology*, vol. 34, no. 1, pp. 83-90.

Vikesland, PJ, Valentine, RL & Ozekin, K 1996, 'Application of product studies in the elucidation of chloramine reaction pathways', *Water Disinfection and Natural Organic Matter*, vol. 649, pp. 105-14.

Vilve, M, Hirvonen, A & Sillanpää, M 2007, 'Ozone-based advanced oxidation processes in nuclear laundry water treatment', *Environmental technology*, vol. 28, no. 9, pp. 961-8.

Vilve, M, Törönen, T & Sillanpää, M 2008, 'Ozonation for the degradation of organic compounds from nuclear laundry water', *Ozone: Science and Engineering*, vol. 30, no. 4, pp. 256-62.

Waara, K-O & Wilander, A 1985, 'Effects of heavy metals on nitrification studied with chemostat technique: With 3 figures and 2 tables in the text', *Internationale Vereinigung für theoretische und angewandte Limnologie: Verhandlungen*, vol. 22, no. 4, pp. 2378-82.

Wang, H, Edwards, MA, Falkinham, JO & Pruden, A 2012, 'Molecular survey of occurrence of Legionella spp., Mycobacterium spp., Pseudomonas aeruginosa and amoeba hosts in two chloraminated drinking water distribution systems', *Applied and Environmental Microbiology*, pp. AEM. 01492-12.

Wang, H, Hu, C, Hu, X, Yang, M & Qu, J 2012, 'Effects of disinfectant and biofilm on the corrosion of cast iron pipes in a reclaimed water distribution system', *water research*, vol. 46, no. 4, pp. 1070-8.

Wang, H, Keller, AA & Li, F 2010, 'Natural organic matter removal by adsorption onto carbonaceous nanoparticles and coagulation', *Journal of Environmental Engineering*, vol. 136, no. 10, pp. 1075-81.

Wang, H, Shen, Y, Hu, C, Xing, X & Zhao, D 2018, 'Sulfadiazine/ciprofloxacin promote opportunistic pathogens occurrence in bulk water of drinking water distribution systems', *Environmental Pollution*, vol. 234, pp. 71-8.

Wang, Y, Ma, L, Mao, Y, Jiang, X, Xia, Y, Yu, K, Li, B & Zhang, T 2017, 'Comammox in drinking water systems', *water research*, vol. 116, pp. 332-41.

Wang, Z-P & Zhang, T 2010, 'Characterization of soluble microbial products (SMP) under stressful conditions', *water research*, vol. 44, no. 18, pp. 5499-509.

Wassink, J, Andrews, R, Peiris, R & Legge, R 2011, 'Evaluation of fluorescence excitation–emission and LC-OCD as methods of detecting removal of NOM and DBP precursors by enhanced coagulation', *Water Science and Technology: Water Supply*, vol. 11, no. 5, pp. 621-30.

Watson, S, Bock, E, Harms, H, Koops, H & Hooper, A 1989, 'Nitrifying bacteria', *Bergey's manual of systematic bacteriology*, vol. 3, pp. 1808-34.

White 2010, *White's Handbook of Chlorination and Alternative Disinfectants* 0196-6006, Black & Veatch Corporation

White, G 1999, 'Chlorination of potable water', *Handbook of chlorination and alternative disinfectants 4th ed. New York, NY: John Wiley & Sons*, pp. 331-536.

WHO 2003, *Silver in drinking-water. Background document for preparation of WHO Guidelines for drinking-water quality*, World Health Organization (WHO/SDE/WSH/03.04/14), Geneva

WHO 2004, *Guidelines for drinking-water quality: recommendations*, vol. 1, World Health Organization.

Wilczak, A, Jacangelo, JG, Marcinko, JP & Odell, LH 1996, 'Occurrence of nitrification in chloraminated distribution systems', *American Water Works Association. Journal*, vol. 88, no. 7, p. 74.

Williams, M, Domingo, J, Meckes, M, Kelty, C & Rochon, H 2004, 'Phylogenetic diversity of drinking water bacteria in a distribution system simulator', *Journal of applied microbiology*, vol. 96, no. 5, pp. 954-64.

Wingender, J, Neu, TR & Flemming, H-C 1999, 'What are bacterial extracellular polymeric substances?', in *Microbial extracellular polymeric substances*, Springer, pp. 1-19.

Neu, TR, Flemming, H-C & Wingender, J 2012, *Microbial extracellular polymeric substances: characterization, structure and function*, Springer Science & Business Media.

Wolfe, RL & Lieu, NI 2003, 'Nitrifying bacteria in drinking water', *Encyclopedia of Environmental Microbiology*.

Wolfe, RL, Lieu, NI, Izaguirre, G & Means, EG 1990, 'Ammonia-oxidizing bacteria in a chloraminated distribution system: seasonal occurrence, distribution and disinfection resistance', *Applied and Environmental Microbiology*, vol. 56, no. 2, pp. 451-62.

Wolfe, RL, Means III, EG, Davis, MK & Barrett, SE 1988, 'Biological nitrification in covered reservoirs containing chloraminated water', *Journal-American Water Works Association*, vol. 80, no. 9, pp. 109-14.

Wonoputri, V, Gunawan, C, Liu, S, Barraud, N, Yee, LH, Lim, M & Amal, R 2015, 'Copper complex in poly (vinyl chloride) as a nitric oxide-generating catalyst for the control of nitrifying bacterial biofilms', *ACS applied materials & interfaces*, vol. 7, no. 40, pp. 22148-56.

Woolschlager, J, Rittmann, B, Piriou, P, Kiene, L & Schwartz, B 2001, 'Using a comprehensive model to identify the major mechanisms of chloramine decay in distribution systems', *Water Science and Technology: Water Supply*, vol. 1, no. 4, pp. 103-10.

Wu, H, Zhang, J, Mi, Z, Xie, S, Chen, C & Zhang, X 2015, 'Biofilm bacterial communities in urban drinking water distribution systems transporting waters with different purification strategies', *Applied microbiology and biotechnology*, vol. 99, no. 4, pp. 1947-55.

Xue, Z, Hessler, CM, Panmanee, W, Hassett, DJ & Seo, Y 2013, 'Pseudomonas aeruginosa inactivation mechanism is affected by capsular extracellular polymeric substances reactivity with chlorine and monochloramine', *FEMS microbiology ecology*, vol. 83, no. 1, pp. 101-11.

Yahya, M, Gerba, C & Rose, J 1994, 'Efficacy of a combined system of copper and silver and free chlorine for inactivation of Naegleria fowleri amoebas in water', *Proc. Intl. Assn. on Water Quality & Health-Related Water Microbiol. Intl. Sym. Budapest, Hungary*.

Yahya, MT, Straub, TM, Gerba, CP & Margolin, AB 1991, 'Inactivation of Bacteriophage MS-2 and poliovirus in copper, galvanized and plastic domestic water pipes', *International Journal of Environmental Health Research*, vol. 1, no. 2, pp. 76-86.

Yapsaklı, K, Çeçen, F, Aktaş, Ö & Can, ZS 2009, 'Impact of surface properties of granular activated carbon and preozonation on adsorption and desorption of natural organic matter', *Environmental Engineering Science*, vol. 26, no. 3, pp. 489-500.

Zhang, Y & Edwards, M 2009, 'Accelerated chloramine decay and microbial growth by nitrification in premise plumbing', *American Water Works Association. Journal*, vol. 101, no. 11, p. 51.

Zhang, Y, Love, N & Edwards, M 2009, 'Nitrification in drinking water systems', *Critical reviews in environmental science and technology*, vol. 39, no. 3, pp. 153-208.

Zhang, Y, Zhao, X, Zhang, X & Peng, S 2015, 'A review of different drinking water treatments for natural organic matter removal', *Water Science and Technology: Water Supply*, vol. 15, no. 3, pp. 442-55.

Zhao, B, Huang, C, Chen, S, Wang, D, Li, T & Qu, J 2011, 'Fouling analysis of polysulfone ultrafiltration membranes used for drinking water treatment', *Water Science and Technology: Water Supply*, vol. 11, no. 6, pp. 668-74.

Zheng, Q, Yang, X, Deng, W, Le, XC & Li, X-F 2016, 'Characterization of natural organic matter in water for optimizing water treatment and minimizing disinfection by-product formation', *Journal of Environmental Sciences*, vol. 42, pp. 1-5.

Zhou, S, Xu, F, Li, S, Song, R, Qi, S, Zhang, Y & Bao, Y 1997, 'Major origin of mutagenicity of chlorinated drinking water in China: humic acid or pollutants', *Science of The Total Environment*, vol. 196, no. 3, pp. 191-6.

Zularisam, A, Ismail, A, Salim, M, Sakinah, M & Matsuura, T 2009, 'Application of coagulation–ultrafiltration hybrid process for drinking water treatment: optimization of operating conditions using experimental design', *Separation and Purification Technology*, vol. 65, no. 2, pp. 193-210.

Appendix A

A.1 Evidence of chloramine decaying soluble microbial products (SMP) accelerating chloramine decay in filtered severely nitrifying water

Results extracted from Krishna, Sathasivan and Sarkar (2012) demonstrated cSMP activity in severely nitrifying water samples. In their experiment, severly nitrified bulk water samples were collected from the reactor and filtered through 0.2µm polycarbonate membranes. Total chlorine, TAN, nitrite and pH were adjusted in a similar way to this experiment whenever the rechloramination was carried out. In nitrified sample, TAN and nitrite reduction were higher than that in the feed water sample (Fig. S1). The ΔTAN (0.100 ± 0.03 mg.L^{-1}) and ΔNO$_2$-N (0.05 mg.L^{-1}) could be due to the influence of cSMP on auto-decomposition and nitrite oxidation, respectively.
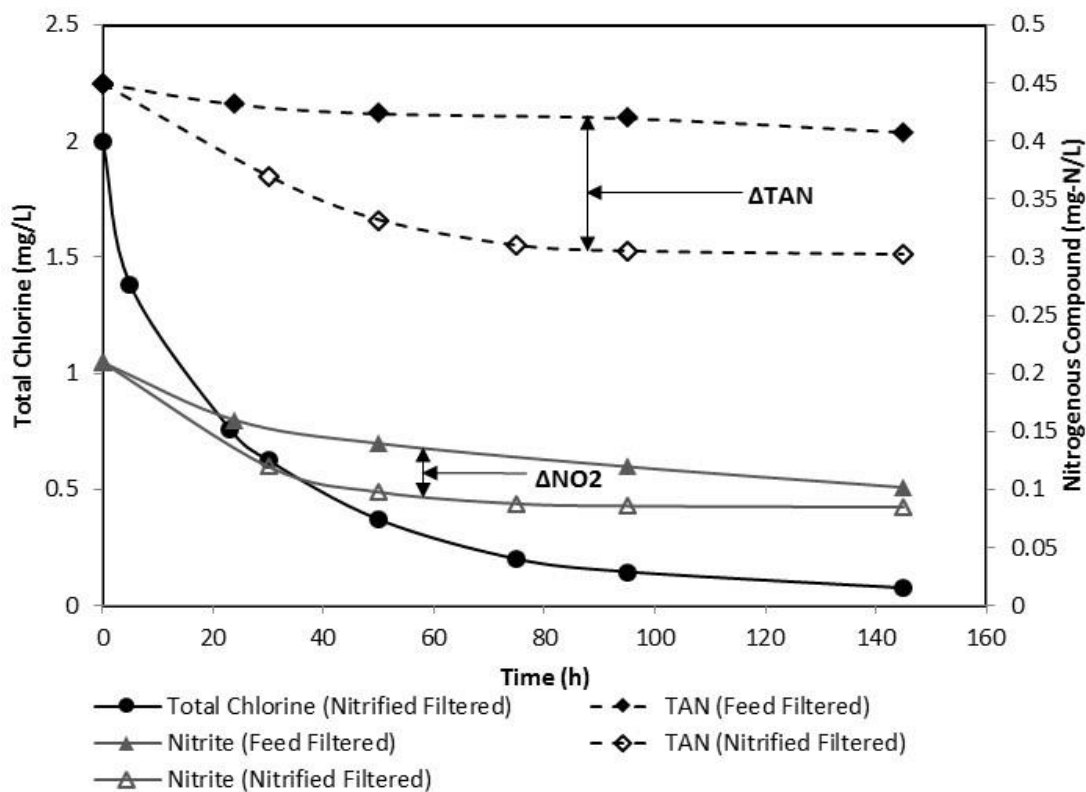
Figure A.1: *Evidence of SMP activity on accelerating auto-decomposition (ΔTAN) and nitrite oxidation (ΔNO$_2$-N)*

A.2 Behaviour of repeatedly re-chlorinated samples obtained from severely nitrified reactor

Keeping the first dosing to the severely nitrified sample as in A.1 (Fig. A.1), the sample was subjected to repeated dosing by Krishna, Sathasivan and Sarker (2012) to test how chloramine decay characteristics change under these conditions. Before each dosing, the nitrite and pH were adjusted and same chloramine was dosesd. The results are shown in Fig. A.2.
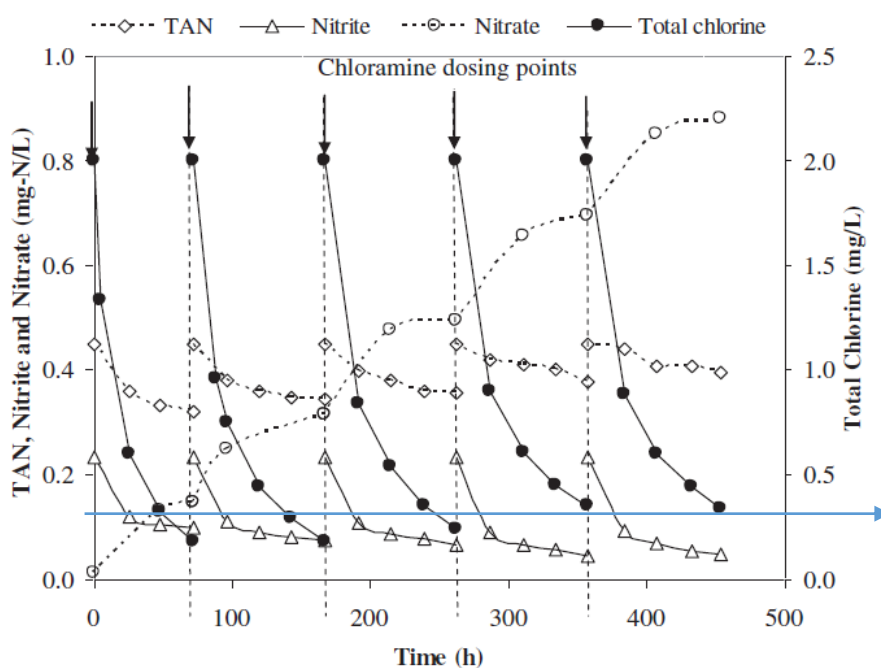

Figure A.2: Total chlorine decay and nitrogenous species profiles at repeated re-chloraminationin the sample collected from the reactor filtration. In each re-chloramination, nitrite, Cl-To-TAN ration, pH and Cl were adjested the same as the foirst one. The vertical arrows show the rechloramination points (Adopted from Bal Krishna et al., 2012).

Similar to the results observed in our experiment, an accelerated drop of TAN and nitrite levels along with rapid chlroramine decay upon consecutive re-chlorination were recorded in their experiment. After each rechloramination TAN loss in the first 25 h was slightly reduced in concert with the slightly stable chloramine. Nitrite loss however increased, probably due to increased concentrations of chloramine. The authors could not explain this observation at that time. Nevertheless, those results support the theory of two different types of SMP (proteins) or two sites of a single protein playing different roles; auto-decomposition and nitrite oxidation, although this needs further exploration in the future.

A.3 DOC variation in the feed water with time

Table A.1: The variation of DOC concentration in the feed water (2014)

Month	Average DOC level (mg-C.L^{-1})
January	7.92
February	6.83
March	5.96
April	5.41
May	4.12
Jun	4.00
July	4.24
August	4.62
September	5.10
October	5.55
November	6.42
December	7.10

The feed water DOC concentrations that were collected from the Orchard Hills water filtration plant varies according to the season of the year. Higher DOC concentrations (7-8 mg-C.L^{-1}) were prominent in the water samples collected from December to February (summer time), which could be due to the heavy rain in that time of the year. Water collected in the winter season normally resulted in low DOC concentrations (4-5 mg-C.L^{-1}) compared to the water collected in summer time.

Appendix B

B.1 Silver inhibition on SMP

Krishna, Sathasivan and Sarker (2012) recently demonstrated that chloramine decaying substances which is released or produced in the nitrified chloraminated systems accelerates chloramine decay. However; applying protein denaturing techniques such as silver addition (presented in Fig. B.1 and B.2) completely inhibit the accelerated chloramine loss.
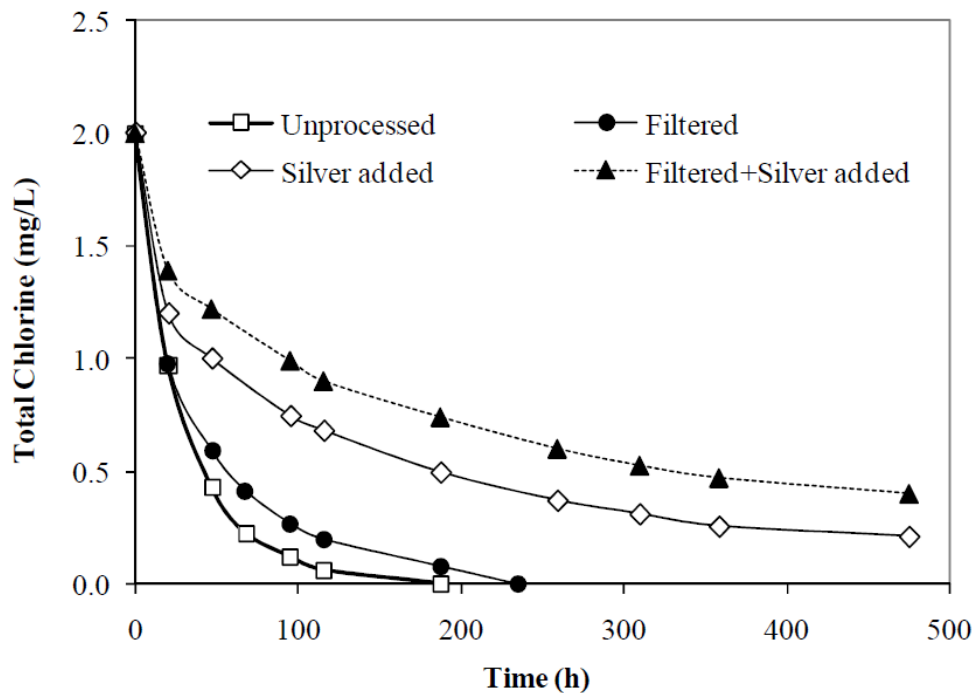

Figure B.1: Chloramine decay profiles in severely nitrified water samples after addition of silver and without addition. 'Unprocessed' represents the not filtered samples where 'filtered' represents the filtered samples through 0.22 μm.

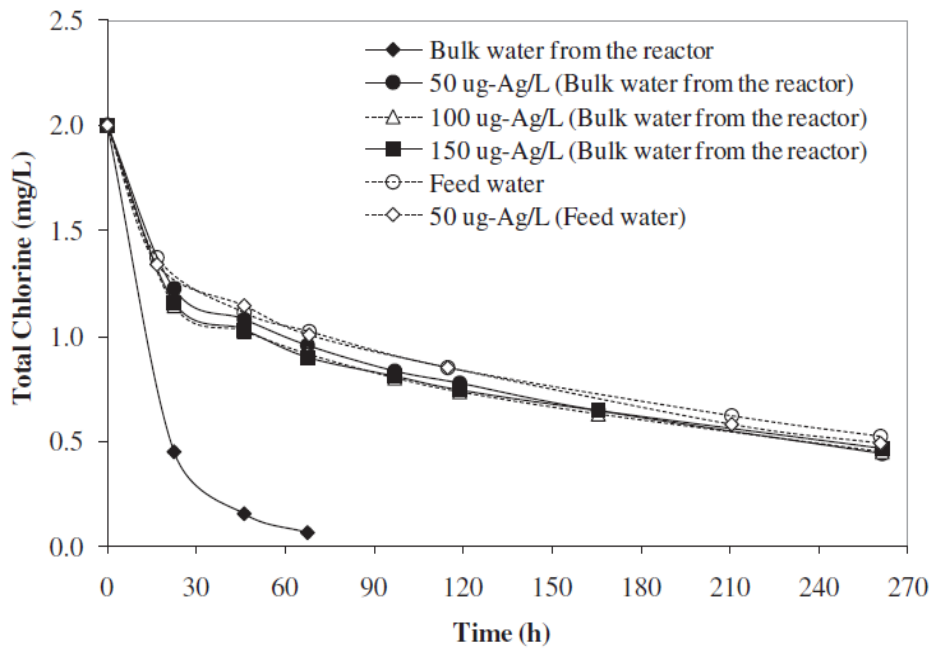(Adapted from Bal Krishna et al. 2012)

Figure B.2: Total chlorine decay profiles in the filtered bulk water collected from the reactor and feed waters after application of silver and adjustment of similar initial conditions in all samples. Initial condition: Total chlorine, 2.0 mg.L^{-1}; TAN, 0.45 mg.L^{-1}; nitrite, 0.21 mg-N.L^{-1}; pH, 8.0; and temperature 20 °C. 7a: application of silver.

(Adapted from Bal Krishna et al. 2012)

Fig. B.2 demonstrates that the chloramine decay profile of the reactor bulk water was similar to the feed water after the addition of silver. All concentrations of silver reduced the observed acceleration present in the reactor bulk water. The minimum dose of 50 μg-Ag.L^{-1} was sufficient to improve the chloramine decay rate.